PCT/DK 2004/000001

Kongeriget Danmark

Patent application No.: PA 2003 00008

Date of filing: 07 January 2003

Applicant: (Name and address)
Symphogen A/S
Elektrovej, Bygning 375
DK-2800 Lyngby
Denmark

Title: A method for manufacturing a recominant polyclonal protein.

IPC: -

This is to certify that the attached documents are exact copies of the above mentioned patent application as originally filed.

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Patent- og Varemærkestyrelsen
Økonomi- og Erhvervsministeriet

28 January 2004

John Nielsen

PATENT- OG VAREMÆRKESTYRELSEN

A method for manufacturing a recombinant polyclonal protein

Field of the invention

The present invention forms the basis of a technology platform for producing recombinant polyclonal proteins, preferably polyclonal antibodies, to be used as a new class of therapeutic antibodies, in the treatment of various infections, inflammatory diseases, transplantation rejection, cancer or allergies.

Background of the invention

A number of infectious diseases or cancer lacks efficient therapies. Monoclonal antibodies have generally not been successful against these targets, partly due to variability of the complex targets and adaptive mutations of target proteins causing immune escape from monoclonal antibody recognition. Polyclonal antibodies on the other hand are able to target a plurality of dynamic targets, e.g. on viruses or cancer cells. Also, polyclonal antibodies have the highest probability of retaining activity in the event of antigenic mutation.

Today, different commercially available polyclonal antibody therapeutics exist including: 1) normal human immunoglobulin isolated from the blood of normal human donors; 2) human hyperimmune immunoglobulin derived from the blood of individual human donors carrying antibodies against a particular disease target, e.g. a virus, which they previously have encountered; and 3) animal hyperimmune immunoglobulin derived from the blood of immunized animals.

Immunoglobulin purified from human blood has proved effective against infections with hepatitis B virus, respiratory syncytial virus, cytomegalovirus and other herpes viruses, rabies virus, botulinum toxin, etc, as well as in the neonatal rhesus D prophylaxis. Immunoglobulin purified from the blood of rabbits immunized with human T cells is used to afford T cell immunosuppression in the treatment or prevention of transplant rejection (e.g.

Thymoglobulin). Normal human immunoglobulin has been utilized to boost the immune system of immunodeficient patients, as well as in the therapy of various autoimmune disorders.

Nevertheless, widespread immunoglobulin use has been limited due to the constrained supply of donor blood raw material, problems with batch-to-batch variations, and variable safety. Animal-derived immunoglobulins in particular are faced with the same problems of immunogenicity as was observedfor animal-derived monoclonal antibodies in the 1980s and 1990s. Finally, as with other blood products, the risk of transmission of infectious agents such as HIV, herpes or hepatitis viruses or prions remains. Accordingly, while clinicians acknowledge that polyclonal antibodies are a preferred therapeutic in some situations, their use has been very limited.

New approaches to generate human immunoglobulins arose with the transgenic animal techniques. Transgenic mice carrying human immunoglobulin loci have been created (US 6,111,166). These mice produce fully human immunoglobulins, thus antibodies against a specific target can be raised by usual immunization techniques. However, larger antibody yields are limited due to the relatively small size of mice. Larger animals have also been made transgenic for the human immunoglobulin genes, e.g. such as cows, sheep, rabbits, and chickens (Kuroiwa, Y. et al. Nature Biotechnology; 2002; 20: 889-893). However, producing polyclonal antibodies for therapy from the blood of such animals is not without complications. First of all, the immunophysiology of the animal and humans may display considerable differences, resulting in difference in the resulting immune repertoire, functional rearrangement, and diversity of the antibody response. Secondly, mitotic instability of the introduced immunoglobulin loci might influence the long-term production of antibodies. A third challenge is to delete the animal's own immunoglobulin loci so that e.g. the animal antibody production will not exceed the production of human antibody. The fourth and probably largest drawback with human antibodies produced in animals is the risk of transmission of infectious agents such as viruses, prions or other pathogens.

Accordingly, there is a need for manufacturing technologies for producing recombinant polyclonal proteins such as antibodies in sufficiently large amounts and with minimal batch-to-batch variations for safe clinical uses. Efficient methods for manufacturinghomogenous recombinant proteins using eukaryotic (in particular mammalian) expression cell lines have been developed for the production of a variety of proteins including monoclonal antibodies, interleukins, interferons, tumor necrosis factor, coagulation factors VII, VIII and IX. Many of these techniques are based on transfection and random integration of the gene of interest into the genome of the expression cell line, followed by selection, amplification and characterization of a high producer expression clone and propagation of this clone as a master expression cell line.

The expression of an inserted foreign gene may be influenced by "position effects" from surrounding genomic DNA. In many cases, the gene is inserted into sites where the position effects are strong enough to inhibit the synthesis of the product of the introduced gene. Furthermore, the expression is often unstable due to silencing mechanisms (i.e. methylation) imposed by the surrounding chromosomal host DNA.

However, conventional transfection and recombinant protein expression techniques using random integration are undesirable for the production of a recombinant polyclonal protein, due to the random nature of the process causing the number and positions of the integrated nucleic acid sequences to vary from cell to cell. Thus, if recombinant polyclonal antibodies are produced by such traditional protocols, it is likely to result in a heterogeneous cell culture with variable expression rates of individual antibodies, and genetic instability due to positional effects of the integrated DNA.

Moreover, antibodies consist of four polypeptide chains, two light and two heavy chains, that are covalently linked during intracellular processing of the antibody molecule. Conventional transfection technology resulting in random integration can lead to the introduction of several copies of different heavy and light chains in the same cell, which would result in random combinations of heavy and light chains, so-called V_H-V_L chain scrambling. Consequently this would deteriorate the performance of the expressed antibodies by causing loss of affinity

and/or specificity, the possible occurrence of new specificities and/or reduced specific activity.

Systems allowing integration and expression of a gene of interest in mammalian cells at a specific genomic location have been developed for the expression of a homogenous recombinant protein composition (US 4,959,317; US 5,654,182; WO 98/41645; WO 01/07572). Especially WO 98/41645 describes the site-specific integration for production of a mammalian cell line that secretes, for example, immunoglobulin. However this expression is monoclonal and there is no indication that transfections could be done with a library of vectors. Nor are there any suggestions how to maintain the original diversity generated by specific V_H-V_L combinations in a library.

Disclosure of contribution

The present invention provides solutions for the generation of a cell line for manufacturing a recombinant polyclonal protein avoiding biased expression of the recombinant polyclonal antibody. Further it does not utilize animals in the production thereby omitting ethical as well as clinical reservations.

Description of the drawings

Figure 1: Schematic representation of a "head-to-head promoter" expression vector comprising the following elements: Amp pro= A promoter allowing expression of the ampicillin resistance gene. Amp= An ampicillin resistance gene. pUC origin= A pUC origin of replication. Restriction enzyme sites: *Not*I and *Eco*RI. Promoter A/Promoter B= head-to-head promoter cassette including leader sequences (e.g. CMV/ MPSV). V Heavy= Sequence coding for the variable heavy chain of a GOI. Constant H= Sequences coding for the constant heavy chain (e.g. the sequences for mouse IgG1 or IgG2B constant heavy chain). R-B-globin pA= Rabbit β-globin poly A sequence. BGH polyA= Bovine Growth Hormone poly A sequence. V Kappa= Sequence coding for the variable kappa of a GOI. Constant Kappa light

chain= Sequence coding for the constant kappa chain. FRT site= A FRT recombination site. Hygromycin= gene for hygromycin resistance. SV40 poly A= poly A signal sequence.

Figure 2: Schematic representation of an expression vector for uni-directional expression comprising the following elements: Amp pro= A promoter allowing expression of the ampicillin resistance gene. Amp= An ampicillin resistance gene. pUC ori=A pUC origin of replication. Promoter A= mammalian promoter including leader sequences (e. g. AdMLP). V Heavy= Sequence coding for the variable heavy chain of a GOI. Constant Heavy chain= Sequences coding for the constant heavy chain (e.g. the sequences for mouse IgG1 constant heavy chain). hGH poly A= Human growth hormone poly A sequence. bGH polyA= Bovine Growth Hormone poly A sequence. V Kappa= Sequence coding for the variable kappa light chain of a GOI. Constant Kappa= Sequence coding for the constant kappa chain. FRT= A FRT recombination site. Hygromycin= gene for hygromycin resistance. SV40 poly A= poly A signal sequence. The sequences of hGH poly A and promoter A could be replaced by an IRES structure.

Figure 3: Flow chart illustrating the generation of a mammalian expression vector.
(A). A schematic representation of a phagemid vector, pSymvc10, which carries a sequence encoding a member of the GOI. P tac and P lacZ = bacterial head-to-head promoter cassette. V kappa = sequence encoding a variable kappa light chain of a GOI. Constant Kappa light chain= Sequence coding for the mouse constant kappa chain. V heavy = a sequence encoding a variable heavy chain of a GOI. Constant heavy chain = Sequence coding for the constant heavy chain CH1 domain. Restriction enzyme sites: *Eco*RI, *Not*I, *Sac*I and *Xho*I. cpIII = phage protein III. Amp pro= A promoter allowing expression of the ampicillin resistance gene. Amp= An ampicillin resistance gene. pUC ori(gin)=A pUC origin of replication.

Step 1: By restriction digestion with *Sac*I and *Xho*I, the bacterial promoter cassette can be excised from pSymvc10 and by ligation, replaced with a mammalian promoter cassette (B) that has also been prepared by restriction digestion with *Sac*I and *Xho*I.

(C) Schematic representation of a phagemid vector, pSymvc12, carrying sequences from the GOI, after promoter exchange with a mammalian head-to-head promoter cassette. Promoter A/Promoter B = head-to-head promoter cassette of choice (e.g. CMV/MPSV). V kappa = sequence encoding for a variable kappa light chain of a GOI. Constant Kappa light chain= Sequence coding for the mouse constant kappa chain. V heavy = sequence encoding for a variable heavy chain of a GOI. Constant heavy chain = Sequence coding for the constant heavy chain CH1 domain. Restriction enzyme sites: *Not*I, *Sac*I, *Xho*I and *Eco*RI. cpIII = phage protein III. Amp pro= A promoter allowing expression of the ampicillin resistance gene. Amp= An ampicillin resistance gene. pUC Ori=A pUC origin of replication.

Step 2: By restriction digestion of pSymvc12 with *Eco*RI and *Not*I, a nucleic acid fragment containing the whole of the kappa, promoter cassette and V heavy can be excised from pSymvc12 and ligated into an isotype-encoding vector, for example pSymvc20, that has also been prepared by restriction digestion with *Eco*RI and *Not*I, thereby generating the mammalian expression vector pSymvc21 (E).

(E) Schematic representation of a mammalian expression vector, pSymvc21, with the variable heavy and kappa regions from the GOI, for antibody expression. This mammalian expression vector comprises the following elements: Amp pro= A promoter allowing expression of the ampicillin resistance gene. Amp= An ampicillin resistance gene. pUC ori(gin)=A pUC origin of replication. Restriction enzyme sites: *Not*I and *Eco*RI. Promoter A/Promoter B = head-to-head promoter cassette of choice (e.g. CMV/MPSV). V kappa = V kappa sequence encoding for a variable kappa light chain of a GOI. Constant Kappa light chain= Sequence coding for a mammalian constant kappa chain (e.g. a mouse constant kappa chain). V heavy = V heavy sequence coding for a variable heavy chain of a GOI. Constant heavy chain= Sequences coding for a mammalian constant heavy chain (e.g. the sequences for mouse IgG1 or IgG2B constant heavy chain). R-B-globin pA= A Rabbit β-globin poly A sequence. BGH poly A= Bovine Growth Hormone poly A sequence. FRT site = A FRT recombination site. Hygromycin= gene for hygromycin resistance. SV40 poly A= SV40 poly A sequence sequence.

Naturally, the order of steps 1 and 2 can be reversed such that a fragment from pSymvc10 containing the whole of the kappa, bacterial promoter cassette and V heavy can be excised from pSymvc10 using *Eco*RI and *Not*I restriction digestion, which can then be ligated into an isotype-encoding vector, for example pSymvc20. The promoter exchange can then be performed on pSymvc20 by restriction digest using *Sac*I and *Xho*I and ligation with a *Sac*I + *Xho*I digested mammalian promoter cassette fragment, for example such as Figure 3B.

Figure 4: Histogram showing the genotype distribution in TG1 cells transformed with Plasmid Preparation 1. Em 223-228 refer to vectors with bacterial promoters encoding anti-β_2-microglobulin (anti-B2M), anti-alkaline phosphate (anti-AP), anti-ovalbumin (anti-OVA), anti-Factor VIII (anti-FVIII), anti-lysozyme (anti-LYS), anti-haptoglobin (anti-HAP), respectively. Em223-228 are vectors of the pSymvc10-type. The number of individual genotypes resembled by the fragment pattern determined by RFLP corresponds to the number of individual colonies among the total number of picked colonies.

Figure 5: Histogram showing the genotype distribution in TG1 cells transformed with Plasmid Preparation 2. Em 229-234 refer to vectors with mammalian promoters (CMV/MPSV) encoding anti-β_2-microglobulin (anti-B2M), anti-alkaline phosphate (anti-AP), anti-ovalbumin (anti-OVA), anti-Factor VIII (anti-FVIII), anti-lysozyme (anti-LYS), anti-haptoglobin (anti-HAP), respectively. Em 229-234 are vectors of the pSymvc12-type. The number of clones represents the number of clones observed that resemble the sequence pattern determined by RFLP of that Em-type (a complete sequence analysis has not been carried out).

Figure 6: Histogram showing the genotype distribution in TG1 cells transformed with Plasmid Preparation 3. Em 235-240 refer to a mouse IgG1 mammalian expression vector (including a rabbit β-globin poly A signal) and encoding anti-β_2-microglobulin (anti-B2M), anti-alkaline phosphate (anti-AP), anti-ovalbumin (anti-OVA), anti-Factor VIII (anti-FVIII), anti-lysozyme (anti-LYS), anti-haptoglobin (anti-HAP), respectively. Em235-240 are vectors of the pSymvc21-type. The number of clones represents the number of clones observed that resemble the sequence pattern determined by RFLP of that Em-type (a complete sequence

analysis has not been carried out).

Figure 7: Histogram showing the genotype distribution in TG1 cells transformed with double digestion/ligation Plasmid Preparation (mass transfer into the mammalian expression vector without DNA amplification in *E. coli* after Plasmid Preparation 1 step).

Figure 8: Histograms showing the genotype distribution in CHO-Flp-In cells transfected with a mixture of mammalian expression vectors encoding the six genes of interest at A) day 16 and B) day 34 post-transfection.

Figure 9: Antigen-specific ELISA of supernatants derived from CHO-Flp-In cells 34 days after transfection with a mixture of expression vectors encoding the six genes of interest.

Figure 10: Anti-kappa coat ELISA of supernatants derived from pools of CHO-Flp-In cells 34 days after transfection either with a single expression vector encoding one gene of interest or a mixture of expression vectors encoding the six genes of interest.

Description of the invention

The present invention provides methods for producing a recombinant manufacturing cell line for the production of a recombinant polyclonal protein. The present invention allows for the commercial production of a recombinant polyclonal protein for use in pharmaceutical compositions. One important feature of the invention is that during the manufacturing process biased expression of the individual molecules constituting the polyclonal protein is kept at a non-significant level, since this could lead to unwanted batch-to-batch variation. One embodiment of the present invention relates to a method for manufacturing of a recombinant polyclonal protein of interest, said method comprising (a) providing a collection of cells, wherein a majority of individual cells comprises one nucleic acid molecule encoding a distinct variant member of a recombinant polyclonal protein of interest, said nucleic acid molecule being integrated into the same site of the genome of each individual cell, (b) culturing said collection of cells under suitable conditions for expression of a recombinant polyclonal

protein of interest, and (c) obtaining the expressed recombinant polyclonal protein from the cells or culture media.

In further embodiments of the present invention the collection of cells, which collectively expresses a recombinant polyclonal protein of interest, is obtainable by introducing a library of vectors of interest into a collection of cells by (a) transfecting cells with a library of vectors of interest for site specific integration, wherein each member of said library of vectors comprises one nucleic acid molecule encoding one member of a recombinant polyclonal protein of interest, and (b) optionally selecting for cells comprising an integrated copy from said library of vectors.
Alternatively, the collection of cells is obtainable by (a) transfecting separate aliquots of cells with each member of a library of vectors of interest, wherein each member of said library of vectors comprises one nucleic acid molecule encoding one member of a recombinant polyclonal protein of interest, (b) optionally selecting for cells comprising an integrated copy from said library of vectors of interest, and (c) pooling the transfected cells to form a collection of cells, which collectively expresses the recombinant polyclonal protein of interest.

In a preferred embodiment of the present invention, the selection step for cells having an integrated copy from the library of the vectors of interest for site specific integration is performed.

In a further embodiment of the invention the nucleic acid molecules of interest encode multiple members of a recombinant polyclonal protein and are preferably integrated into a pre-selected locus that mediates high-level expression, a so-called hot spot, thereby creating high producer cell lines.

In other embodiments, are nucleic acid molecules of interest operably linked to a heterologous regulatory element capable of expressing said nucleic acid molecule in a constitutive or inducible manner, even more preferred this heterologous regulatory element results in high-

level expression. The nucleic acid molecules of interest collectively encode the recombinant polyclonal protein composition.

In one embodiment of the invention the individual members of a polyclonal protein comprises one, two, or more polypeptide chains. In a preferred embodiment, the polyclonal protein is constituted of antibodies or fragments thereof, for example Fab or Fv fragments.
In a further embodiment of the invention the polyclonal antibody or fragment thereof comprise sequence differences between the individual antibody molecules in the variable region (V region) or in the constant region (C region) or both.

In a further embodiment of the invention are the host cells used for transfection preferably mammalian cells, such as Chinese hamster ovary (CHO) cells, COS cells, BHK cells, myeloma cells (e.g. Sp2/0 cells, NS0), NIH 3T3, fibroblast or immortalized human cells such as HeLa cells, HEK 293 cells or PER.C6. Most preferably are CHO cells, however, non-mammalian eukaryotic or prokaryotic cells, such as plant cells, insect cells, yeast cells, bacteria, fungi etc., can also be employed.

Yet another embodiment of the invention is a pharmaceutical composition comprising as an active ingredient a recombinant polyclonal protein for example obtainable by the methods according to the invention. The recombinant polyclonal protein of the composition is specific for or reactive against a predetermined disease target. Such pharmaceutical compositions can be used for the treatment or prevention of diseases such as cancer, infections, inflammatory diseases, allergy, asthma and other respiratory diseases, autoimmune diseases, cardiovascular diseases, diseases in the central nervous system, metabolic and endocrine diseases, transplant rejection, or undesired pregnancy, in a mammal such as a human, a domestic animal, or a pet.

Pharmaceutical compositions according to the invention can further take effect by reacting with or binding to infectious microorganisms and can be used for treating or preventing diseases caused by bacteria, mycobacteria, viruses, mycoplasma, rickettsia, spirochetes, protozoa, fungi, helminthes, prions and ectoparasites.

Another embodiment of the invention is a method for generating a recombinant manufacturing cell line, wherein a majority of the individual cells are expressing a distinct variant member of a recombinant polyclonal protein of interest from one copy of a nucleic acid molecule of interest, said nucleic acid molecule being integrated into the same site of the genome of the individual cells of said manufacturing cell line.

A further embodiment in generating a recombinant manufacturing cell line constitutes providing a library of vectors of interest, each comprising one copy of a nucleic acid molecule encoding one member of a polyclonal protein and one or more recombinase recognition sequences. The recombinase recognition sequence facilitating site specific integration into the genome of a manufacturing cell line once the library of vectors for site specific integration has been introduced into the collection of cells. In this collection each cell comprises one or more recombinase recognition sequences at specific sites in its genome and ensuring the presence of a suitable recombinase is ensured. Recombinases can be provided to the cell by several method either (i) it is already expressed by the cells into which the nucleic acid molecule of interest is introduced, or (ii) a recombinase encoding sequence is operatively encoded by said vector of interest, or (iii) a recombinase is provided through expression of a recombinase encoding sequence from a second vector, or (iv) it is provided directly to the cell as a protein.

Yet another embodiment of the invention is a recombinant manufacturing cell line comprising a collection of cells, wherein a majority of the individual cells are capable of expressing a distinct variant member of a recombinant polyclonal protein of interest from one copy of a nucleic acid molecule of interest, said nucleic acid molecule being integrated into the same site of the genome of each transfected cell in said collection of cells. The cell line is preferably a mammalian cell line such as Chinese hamster ovary (CHO) cells, COS cells, BHK cells, myeloma cells (e.g. Sp2/0 cells, NS0), NIH 3T3, fibroblast or immortalized human cells such as HeLa cells, HEK 293 cells, or PER.C6. However, non-mammalian eukaryotic or prokaryotic cells, such as plant cells, insect cells, yeast cells, bacteria, fungi etc., can also be employed.

Another embodiment of the present invention is a library of vectors of interest for site specific integration wherein each vector comprises one or more recombinase recognition sequences

and one copy of a nucleic acid molecule of interest encoding a distinct variant member of a recombinant polyclonal protein of interest. Each member of said library of vectors may further comprise a nucleic acid molecule encoding a suitable recombinase. Such a library preferably encodes recombinant polyclonal antibodies.

Definitions

By "protein" or "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification. Proteins can exist as a monomer or multimer, comprising one, two or more assembled polypeptide chains, fragments of proteins, polypeptides, oligopeptides, or peptides.

As used herein, the term "polyclonal protein" or "polyclonality" refers to a protein composition comprising different, but homologous protein molecules. Thus, each protein molecule is homologous to the other molecules of the composition, but also contains one or more stretches of variable polypeptide sequence, which is characterized by differences in the amino acid sequence between the individual members of the polyclonal protein. Known examples of such polyclonal proteins include antibody or immunoglobulin molecules, T cell receptors, B cell receptors and macrophage receptors. A polyclonal protein may consist of a defined subset of protein molecules, which has been defined by a common feature such as the shared binding activity towards a desired target, e.g. in the case of a polyclonal antibody against the desired target antigen.

The term a "naturally variable protein" is used interchangeably with a polyclonal protein.

The term "polyclonal protein of interest" covers a defined polyclonal protein subset, which shares a common feature, e.g. such as binding activity towards a desired target, e.g. in the case of polyclonal antibodies described by the binding activity or specificity against the target antigen, said antigen being one or more of e.g. separate proteins, microorganisms, parasites, cell types, allergens, or carbohydrate molecules, or any other structures, molecules, or substances, which may be the target of specific antibody binding, or mixtures of said antigens.

The terms "one member of a recombinant polyclonal protein composition" or "one member of a recombinant polyclonal protein of interest" denotes one protein molecule having a variable polypeptide sequence that is homologous, but not identical to the variable polypeptide sequences of the other members of the recombinant polyclonal protein composition or recombinant polyclonal protein of interest.

The terms "a distinct variant member of a recombinant polyclonal protein" denotes one protein molecule having a variable polypeptide sequence that is homologous, but not identical to the variable polypeptide sequences of the other members of the recombinant polyclonal protein.

The term "antibody" describes a functional component of serum and is often referred to either as a collection of molecules (antibodies or immunoglobulin) or as one molecule (the antibody molecule or immunoglobulin molecule). An antibody molecule is capable of binding to or reacting with a specific antigenic determinant (the antigen or the antigenic epitope), which in turn may lead to induction of immunological effector mechanisms. An individual antibody molecule is usually regarded as monospecific, and a composition of antibody molecules may be monoclonal (i.e. consisting of identical antibody molecules) or polyclonal (i.e. consisting of different antibody molecules reacting with the same or different epitopes on the same antigen or even on distinct, different antigens). Each antibody molecule has a unique structure that enables it to bind specifically to its corresponding antigen, and all natural antibody molecules have the same overall basic structure of two identical light chains and two identical heavy chains. Antibodies are also known collectively as immunoglobulins. The terms antibody or antibodies as used herein are also intended to include chimeric and single chain antibodies, as well as binding fragments of antibodies, such as Fab or Fv fragments, as well as multimeric forms such as dimeric IgA molecules or pentavalent IgM.

The term "polyclonal antibody" describes a composition of different antibody molecules which is capable of binding to or reacting with several different specific antigenic determinants on the same or on different antigens. Usually, the variability of a polyclonal

antibody is thought to be located in the so-called variable regions of the polyclonal antibody. However, in the context of the present invention, polyclonality can also be understood to describe differences between the individual antibody molecules residing in so-called constant regions, e.g. as in the case of mixtures of antibodies containing two or more antibody isotypes such as the human isotypes IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2, or the murine isotypes IgG1, IgG2a, IgG2b, IgG3, and IgA.

A "recombinant polyclonal antibody of interest" describes a defined recombinant polyclonal antibody subset, which is characterized by the ability to bind to a desired target or desired set of targets, said targets being e.g. a separate protein, a microorganism, a parasite, a cell, an allergen, or a carbohydrate molecule, or another structure, molecule, or substance which may be the target of specific antibody binding, or mixtures thereof.

The term "immunoglobulin" is a collective designation of the mixture of antibodies found in blood or serum.

The term "immunoglobulin molecule" denotes an individual antibody molecule, e.g. as being a part of immunoglobulin, or part of any polyclonal or monoclonal antibody composition.

The term "a library of nucleic acid molecules of interest" is used to describe the collection of nucleic acid molecules, which collectively encode a "recombinant polyclonal protein of interest". When used for transfection, the library of nucleic acid molecules of interest is contained in a library of expression vectors.

As used herein the terms "one copy of a nucleic acid molecule of interest" or "one copy of a gene of interest" are not to be taken literally as a single stretch of nucleic acids, but rather as one copy of all the required nucleic acid molecules, which are necessary to produce all the subunits of one molecule of the protein of interest. Some examples, where more than one nucleic acid molecule usually is required to give rise to a complete molecule of a protein of interest include B cell receptors, antibodies and fragments of antibodies such as Fab's and variable domains, or T cell receptors. When introduced into the cell, the nucleic acid

molecules, which together encode the fully assembled protein of interest molecule, reside in the same vector, thus being linked together in one nucleic acid molecule.

The terms "a gene of interest" and "genes of interest" as used herein, refer to a nucleic acid molecule and nucleic acid molecules, respectively, (genomic or cDNA) that encodes a protein of interest. The term "GOI" is used as an abbreviation of (a) gene(s) of interest.

As used herein, the term "vector" refers to a nucleic acid molecule into which a nucleic acid sequence can be inserted for transport between different genetic environments and/or for expression in a host cell. A vector capable of integrating into the genome of a host cell at a pre-determined, specific locus in the genome is herein named "a vector for site specific integration". If the vector carries regulatory elements for transcription of the nucleic acid sequence inserted in the vector (at least a suitable promoter), the vector is herein called "an expression vector". If the expression vector is capable of integrating at a pre-determined, specific locus in the genome of the host cell, the expression vector is herein called "an expression vector for site specific integration". If the nucleic acid sequence inserted into the above identified vectors encodes a protein of interest as herein defined, the following terms are used "vector of interest", "vector of interest for site specific integration", "expression vector of interest" and "expression vector of interest for site specific integration". The term "an isotype-encoding vector" refers to a vector carrying nucleic acid sequences encoding an antibody isotype. In the present specification, phagemid vector and phage vector are used interchangeable and covers a nucleic acid molecule into which a sequence encoding a recombinant protein has been inserted. The terms "plasmid" and "vector" are used interchangeably. The invention is intended to include such other forms of vectors, which serve equivalent functions for example phagemids and virus genomes or any nucleic acid molecules capable of directing the production of a desired protein in a proper host.

The term "each member of the library of vectors of interest" is used to describe individual vector molecules with a particular nucleic acid molecule derived from a library of vectors of interest, where the nucleic acid molecule encodes for one member of the recombinant polyclonal protein of interest.

The term "mass transfer" is used to describe the transfer of nucleic acid sequences of interest from one population of vectors to another population of vectors and doing so for each DNA simultaneously without resorting to isolation of the individual DNA's of interest. Such populations of vectors can be libraries containing for example variable regions, promoters, leaders or enhancing elements of interest. These sequences can then be moved without prior isolation from for example a phage vector to a mammalian expression vector. Especially for antibody sequences this technique ensures that the linkage between V_H and V_L diversity is not lost while moving libraries from for example a selection vector, e.g. a phage display vector, to a mammalian expression vector. Hereby the original pairing of V_H and V_L is retained.

The term "transfection" is herein used as a broad term for introducing foreign DNA into a cell. The term is meant to cover other functional equivalent methods for introducing foreign DNA into a cell, such as e.g. transformation, infection, transduction or fusion of a donor cell and an acceptor cell.

The term " selection" is used to describe a method where cells are exposed to a cytotoxic agent and only cells having taken up and expressing the selectable marker will survive.

The terms "selectable marker gene", "selection marker gene", and "marker gene" are used to describe a gene encoding a selectable marker (e.g. a gene conferring resistance against some cytotoxic drug such as certain antibiotics) which is co-introduced into the cells together with the gene(s) of interest.

The term "recombinant protein" is used to describe a protein that is expressed from a transfected cell line.

As used herein, the term "operably linked" refers to a segment being linked to another segment when placed into a functional relationship with the other segment. For example, DNA encoding a signal sequence is operably linked to DNA encoding a polypeptide if it is expressed as a leader that participates in the transfer of the polypeptide to the endoplasmic

reticulum. Also, a promoter or enhancer is operably linked to a coding sequence if it stimulates the transcription of the sequence.

The term "a majority of the individual cells" refers to a percentage of the cells such as more than 80 %, preferably more than 85%, more preferably 90%, 95%, or even 99% or higher.

As used herein, the term "genome" is not to be taken literally as the normal complement of chromosomes present in a cell, but also extra-chromosomal elements that can be introduced into and maintained in a cell. Such extra-chromosomal elements can include, but are not restricted to, mini-chromosomes, YACs (Yeast artificial chromosomes), MACs (Mouse artificial chromosomes), or HACs (Human artificial chromosomes).

The term "promoter" refers to a region of DNA involved in binding the RNA polymerase to initiate transcription.

The term "head-to-head promoters" refers to a promoter pair being placed in close proximity so that transcription of two gene fragments driven by the promoters occurs in opposite directions.

An "antibiotic resistance gene" is a gene encoding a protein that can overcome the inhibitory or toxic effect that an antibiotic has on a cell ensuring the survival and continued proliferation of cells in the presence of the antibiotic.

The term "internal ribosome entry site" " or "IRES" describes a structure different from the normal 5' cap-structure on an mRNA. Both structures can be recognized by a ribosome to initiate scanning for an AUG codon to initiate translation. By using one promoter sequence and two initiating AUG's, a first and a second polypeptide sequence can be translated from a single mRNA. Thus, to enable co-translation of a first and a second polynucleotide sequence from a single bi-cistronic message, the first and second polynucleotide sequence can be transcriptionally fused via a linker sequence including an IRES sequence that enables translation of the polynucleotide sequence downstream of the IRES sequence. In this case, a

transcribed bi-cistronic RNA molecule will be translated from both the capped 5' end and from the internal IRES sequence of the bi-cistronic RNA molecule to thereby produce both the first and the second polypeptide.

The term "inducible expression" is used to describe expression that requires interaction of an inducer molecule or the release of a co-repressor molecule and a regulatory protein for expression to take place.

The term "constitutive expression" refers to expression which is not usually inducible.

The term "recombinase" refers to an enzyme that catalyses recombination between two or more recombination sites. Recombinases useful in the present invention catalyze recombination at specific recombination sites that are specific nucleic acid molecules recognized by a particular recombinase.

The term "V_H-V_L chain scrambling" covers the mixing of V_H and V_L gene segments resulting in loss of the original combination of V_H and V_L genes, resulting in novel combinations of V_H and V_L, which might not have any therapeutic effect.

The term "manufacturing cell line" refers to a population of protein expressing cells that are transfected with a library of nucleic acid molecules of interest such that the majority of the individual cells, which together constitute the manufacturing cell line, carry only one copy of a nucleic acid molecule of interest, which encodes one member of the recombinant polyclonal protein of interest, and that each copy is integrated into the same site of the genome of each cell. The cells constituting the manufacturing cell line are selected for their ability to retain the integrated copy of the nucleic acid molecule of interest, for example by antibiotic selection. Cells which can constitute such a manufacturing cell line can be for example bacteria, fungi, eukaryotic cells, such as yeast, insect cells or mammalian cells, especially immortal mammalian cell lines such as CHO cells, COS cells, BHK cells, myeloma cells (e.g. Sp2/0 cells, NS0), NIH 3T3, and immortalized human cells, such as HeLa cells, HEK 293 cells, or PER.C6.

The term "hot spot" as in "hot spot cell line" refers to a pre-established locus of the genome of the cell that has been selected or generated and characterized for highly efficient transcription of an integrated nucleic acid molecule of interest upon integration of the expression vector into that site.

The term "bias" is used to denote the phenomenon during recombinant polyclonal protein production, wherein the composition of a polyclonal vector, polyclonal cell line, or polyclonal protein alters over time due to random genetic mutations, differences in growth kinetics between individual cells, differences in expression levels between different expression construct sequences, or differences in the cloning efficiency of DNA.

The term "RFLP" refers to "restriction fragment length polymorphism", a method whereby the migratory gel pattern of nucleic acid molecule fragments are analyzed after cleavage with restriction enzymes.

The term "HDS" refers to a high density screening method where many discrete molecules are tested in parallel on membranes so that large numbers of test compounds are screened for a given activity simultaneously.

As used herein, "TaqMan PCR" refers to a PCR assay based on the TaqMan system described by Holland, P. M. et al., Proc. Natl. Acad. Sci. U.S.A. 88: 7276-7280 (1991).

The term "5' UTR" refers to a 5' untranslated region of the mRNA.

The term "Pfu PCR" refers to a PCR reaction carried out using a Pfu DNA polymerase (isolated from Pyrococcus furiosus), which is utilized because it has the highest fidelity among known thermostable polymerases.

Abbreviations: "CMV" = (human) Cytomegalo Virus. "MSPSV" = Myeloproliferative Sarcoma Virus. "AdMLP" = Adenovirus Major Late Promoter. SV40 poly A = Simian Virus

40 poly A signal sequence. GFP = Green Flourescent Proteins. PVDF = polyvinylidene difluorid.

Detailed description of the invention

The recombinant polyclonal protein expression system

The present invention provides methods for the manufacturing of recombinant polyclonal proteins including recombinant polyclonal proteins of interest. Technologies for the consistent production of a recombinant polyclonal protein, such as, for example, a recombinant polyclonal antibody are provided. In particular, it is contemplated that this invention will open up the possibility for large-scale manufacturing and production of a new class of therapeutics comprising recombinant therapeutic polyclonal antibodies.

In order to obtain predictable expression of a polyclonal protein from a manufacturing cell line, the regulatory properties of the genomic integration site should be reasonably well understood. Introduction into a predefined genomic site can in principle be achieved by homologous recombination. However, owing to the dominance of illegitimate recombination events, homologous recombination is very inefficient. To circumvent these problems the expression system of the present invention was developed. The system of the present invention ensures that a library of vectors of interest comprising the nucleic acid molecules of interest can be inserted into a pre-characterized chromosomal location by a recombinase-mediated cassette exchange procedure, thereby generating a cell line, wherein a majority of the individual cells expresses a distinct variant member of the recombinant polyclonal protein of interest.

Recombinases such as Cre, Flp, beta-recombinase, Gin, Pin, PinB, PinD, R/RS or phage ΦC31 integrase can be used. Suitable recombinases for integration into the chromosomal location can be provided either (i) by expression from the cell's own genome into which said nucleic acid molecule is introduced, or (ii) operatively encoded by the nucleic acid molecule

inserted into the cell, or (iii) provided through expression from a second nucleic acid molecule, or (iv) provided as a protein.

In a preferable embodiment, the vector of interest is integrated into a locus that mediates high-level transcription and expression of the nucleic acid molecule of interest, a so-called "hot spot".

The host cell line used is preferably a mammalian cell line comprising those typically used for biopharmaceutical protein expression, e.g., CHO cells, COS cells, BHK cells, myeloma cells (e.g. Sp2/0 cells, NS0), NIH 3T3, and immortalized human cells, such as HeLa cells, HEK 293 cells, or PER.C6.In the present invention CHO cells were used. However, a person of ordinary skill in the art would easily be able to substitute CHO cells with other mammalian cells as described, or even utilize other types of cells, including plant cells, yeast cells, insect cells, fungi and bacteria. Thus the choice of cell type is not intended to be limiting to the invention.

In a preferable embodiment, mammalian cells containing a pre-characterized hot spot, mediating high expression levels of the recombinant polyclonal protein of interest are used.

In a further embodiment of the present invention, are nucleic acid molecules of interest are integrated in a site specific manner utilizing the same chromosomal integration site in the host cells, and expressing the same overall protein structure apart from the differences observed in the variable region of the recombinant polyclonal protein of interest, e.g. the antigen-binding region of antibodies. Therefore, a majority of cells within such a pool of cells should display similar characteristics with respect to productivity and genetic stability and hence this technology offers the possibility of a controlled production of a recombinant polyclonal protein, e.g. a recombinant polyclonal antibody.

The recombinant polyclonal protein of the present invention is intended to cover a protein composition comprising different, but homologous protein molecules, which are naturally variable. Thus, each protein molecule is homologous to the other molecules of the composition, but also contains one or more stretches of variable polypeptide sequence, which is characterized by differences in the amino acid sequence between the individual members of the polyclonal protein. Usually, the natural variability of a polyclonal antibody is thought to

be located in the so-called variable regions or V regions of the polyclonal antibody polypeptide chains. However, in the context of the present invention, variability in the polypeptide sequence (the polyclonality) can also be understood to describe differences between the individual antibody molecules residing in so-called constant regions or C regions of the antibody polypeptide chains, e.g. as in the case of mixtures of antibodies containing two or more different antibody isotypes, such as the human isotypes IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2, or the murine isotypes IgG1, IgG2a, IgG2b, IgG3, and IgA. Thus, a recombinant polyclonal antibody may comprise antibody molecules that are characterized by sequence differences between the individual antibody molecules in the variable region (V region) or in the constant region (C region) or both.

A polyclonal protein of interest comprises a defined subset of proteins, which have been defined by a common feature such as the shared binding activity towards a desired target, e.g. in the case of polyclonal antibodies against the desired target antigen.

In mammals, there are several known examples of polyclonal proteins either circulating freely in the blood such as antibodies or immunoglobulin molecules or present on cell surfaces such as T cell receptors and B cell receptors. The diversity of these naturally occurring polyclonal proteins are, in some mammals, achieved by genetic recombination of genes encoding variable regions of these proteins. Antibodies are further known to increase their diversity by somatic mutation. The present invention can utilize these natural diversities by cloning the sequences responsible for the diversity (e.g. in variable domains) before generating a manufacturing cell line expressing such a natural polyclonal protein as a recombinant polyclonal protein.

Diversities of proteins can also be made in an artificial way, for example synthetic or by mutation, either random or point mutation, of a nucleic acid sequence encoding a single protein, thereby generating a polyclonal population of the single protein. Such proteins can be a wide variety of proteins, for example immunoglobulins and T and B cell receptors as described above, but it may also be other proteins such as, but not limited to, receptors, receptor ligands, hormones, ion-channels, pumps and so forth.

In a preferred embodiment of the invention, the recombinant polyclonal protein is a recombinant polyclonal antibody.

In addition to the diversity achieved by the genetic and somatic recombination in the so-called variable regions, there are different isotypes of the immunoglobulins, which are defined by the heavy chain. The main isotypes are IgM, IgG, IgA, IgD, and IgE.

A recombinant polyclonal protein of the present invention can therefore also be constituted of the different isotypes or more preferred of different subclasses. Polyclonality of the immunoglobulins can thus occur in the constant part or in the variable domain of the immunoglobulin or in both the constant part and the variable domain.

Polyclonality in the so-called constant region of the antibodies, especially in the heavy chain, is of interest with regard to therapeutic application of antibodies. First of all, the various immunoglobulin isotypes have different biological functions (summarized in table 1), which it might be desirable to combine when utilizing antibodies for treatment. Thus, the literature supports that different isotypes of immunoglobulin might be implicated in different aspects of natural immune responses (Canfield and Morrison 1991; Kumpel et al 2002; Stirnadel et al 2000) Epidemiol. Infect.124, 153-162).

Table 1: Biological functions of the human immunoglobulin isotypes

	Human Immunoglobulin								
	IgG_1	IgG_2	IgG_3	IgG_4	IgA_1	IgA_2	IgM	IgD	IgE
Classical complement activation	+++	++	++++	+	-	-	++++	-	-
Alternate complement activation	+	+	+	+++	+	-	-	+	-
Placental transfer	+	++	+	++	-	-	-	-	-
Bacterial lysis	+	+	+	+	+++	+++	+	?	?
Macrophage/other phagocytes binding	+	-	+	+	+	+	-	-	-
Mast cell/basophils binding	-	-	-	-	-	-	-	-	-
Staphylococcal Protein A reactivity	+	+	-	+	-	-	-	-	-

The host cell

A suitable host cell comprises in a region of its genome one or more suitable recombination sites, i.e. nucleic acid molecules recognizable by one or more recombinase enzymes. To be able to select for integrants, i.e. cells having an integrated copy of the nucleic acid molecule of interest, in an integration site, the recombination site is operably linked to a first selection gene, e.g. an antibiotic resistance gene, situated 3' of the recombination site. Furthermore, a weak promoter (e.g. a truncated SV40 early promoter) and a transcription start codon may be situated 5' of the recombination site that constitutes an integral part of the resistance marker-coding region. Thus, the transcription start codon initiates the start of transcription of the selection gene in the host cell before transfection with the library of expression vectors encoding the polyclonal protein.

Host cells for site-specific integration as described above can be generated from any cell which can integrate DNA into their chromosomes or retain extra-chromosomal elements such as mini-chromosomes, YACs (Yeast artificial chromosomes), MACs (Mouse artificial chromosomes), or HACs (Human artificial chromosomes). Preferably mammalian cells such

as CHO cells, COS cells, BHK cells, myeloma cells (e.g. Sp2/0 cells, NS0), fibroblasts such as NIH 3T3, and immortalized human cells, such as HeLa cells, HEK 293 cells, or PER.C6, are used.

In one way, the desired cells are obtained by transfection with a randomly integrating plasmid comprising a weak promoter (e.g. a truncated SV40 early promoter), a transcription start codon, a recombination site situated 3' of the start codon. Preferably, the integrating plasmid also comprises a marker gene coupled to a first selection gene. One example of such an integrating plasmid is the pLacZeo2 from Invitrogen. The marker gene can be used to evaluate the relative strength of expression at the genomic location used for inserting a nucleic acid molecule of interest. A marker gene, e.g. beta-galactosidase, GFP or a cell surface marker, can be linked to the first selection gene in a gene fusion or transcriptionally linked by an IRES (internal ribosomal entry site) such that co-expression of the first selection gene and marker gene occurs, allowing evaluation of the relative expression levels from cell line to cell line.

Cell lines, which achieve high expression of the marker gene upon integration of a single copy of the plasmid, are used for transfection with the gene of interest. The recombination site in the host cell is preferably located in a gene or region of particularly active expression, i.e. in a so-called hot spot.

The vector for site-specific integration

A suitable vector comprises a suitable recombination site linked to a suitable selection gene different from the selection gene used for construction of the host cell. Suitable selection genes for use in mammalian cell expression include, but are not limited to, the dihydrofolate reductase gene (DHFR) which can be selected for with methotrexate, the thymidine kinase gene (TK), or prokaryotic genes conferring drug resistance, gpt (xanthine-guanine phosphoribosyltransferase), which can be selected for with mycophenolic acid; neo (neomycin phosphotransferase), which can be selected for with G418 in eukaryotic cell and neomycin or kanamycin in prokaryotic cells.

In one aspect of the present invention, the selectable gene is neither preceded by a promoter nor equipped with a translation initiating codon. If this vector is integrated at a location other than the selected recombination site in the genome of the host cell, no expression of this second selection gene can occur due to lack of promoter and initiation codon. If integration occurs at the selected recombination site in the genome of the host cell, the second selection gene is expressed and expression of the first selection gene is lost.

Integration may e.g. be carried out using a so-called FRT site (5'-gaagttcctattccgaagttcctattctctagaaagtataggaacttc-3') in the genome and on the vector for site specific integration together with the Flp recombinase from *Saccharomyces cerevisiae.* However, other recombinase systems may equally well be used, including those of Cre recombinase and a variety of lox sites such as loxP from bacteriophage P1 or variants or mutants thereof, e.g. lox66, lox71, lox76, lox75, lox43, lox44 and lox511 (C. Gorman and C. Bullock, Curr. Opinion in biotechnology 2000, 11: 455-460) or by using phage integrase ΦC31, which carries out recombination between the attP site and the attB site (A.C. Groth et al. PNAS 2000, 97:5995-6000). Further recombinase systems that could be utilized in the present invention are, but are not limited to, the β recombinase-six system from bacterial plasmid pSM19035, the Gin-gix system from bacteriophage Mu or the R-RS system from Zygosaccharomyces rouxii.

A further variant to the site-specific recombination system is to use non-homologous recombination sites. In such a system, two non-identical recombination sites are introduced into the host genome for the generation of specific target sites. Recombination sites corresponding to those flanking the target site also flank the construct containing the gene of interest. Such a system has been.described in WO 99/25854, which is hereby incorporated by reference in its entirety. The use of non-homologous recombination sites was shown to suppress excision of the GOI from the chromosome. The non-identical recombination sites can be composed of any of the recombination sites described above as long as the corresponding recombinases are provided. For example, non-identical recombination sites could consist of a FRT site and a mutant FRT site utilizing a Flp recombinase for integration or a FRT site and a loxP site utilizing Flp and Cre recombinases for the integration.

Another system that minimizes excision of the GOI after its site-specific integration into the chromosome is the ΦC31 integrase, also mentioned above. This system has been described thoroughly in patent applications WO 01/07572 and WO 02/08409, hereby incorporated by reference in their entirety.

In a further aspect of the invention, the vector for site specific integration of the gene of interest further comprises DNA encoding one member of the recombinant polyclonal protein of interest, optionally preceded by its own mammalian promoter directing expression of the protein. If a member of the recombinant polyclonal protein of interest comprises more than one protein chain, e.g. if the member is an antibody or T cell receptor, the DNA encoding the chains of the protein can be preceded by their own mammalian promoter directing high levels of expression (uni- or bi-directional) of each of the chains. In a bi-directional expression a head-to-head promoter configuration in the expression vector can be used and for a uni-directional expression two promoters or one promoter combined with e.g. an IRES sequence can be used for expression.

A nucleic acid molecule encoding a functional leader sequence can be included in the expression vector to direct the gene product to the endoplasmic reticulum or a specific location within the cell such as an organelle. A strong polyadenylation signal can be situated 3' of the protein-encoding DNA. The polyadenylation signal ensures termination and polyadenylation of the nascent RNA transcript and is correlated with message stability. The DNA encoding a member of the recombinant polyclonal protein of interest can, for example, encode both the heavy and light chains of an antibody or fragments thereof, each gene sequence optionally being preceded by their own mammalian promoter elements and/or followed by strong poly A signals directing high level expression of each of the two chains.

The expression vector for site specific integration can carry additional transcriptional regulatory elements, such as enhancers or UCOE (ubiquitous chromatin opening elements) for increased expression at the site of integration. Enhancers are nucleic acid molecules that interact specifically with cellular proteins involved in transcription. The UCOE opens

chromatin or maintains chromatin in an open state and facilitates reproducible expression of an operably-linked gene (described in more detail in WO 00/05393,hereby incorporated by reference in its entirety). When one or more of the regulatory elements described in the above are integrated into the chromosome of a host cell they are termed heterologous regulatory elements.

Establishing an expression system for high-level expression of proteins

Methods for introducing a nucleic acid molecule into a cell are known in the art. These methods typically include the use of a DNA vector to introduce the sequence into the genome or an extra-chromosomal element. Transfection of cells may be accomplished by a number of methods known to those skilled in the art, including calcium phosphate precipitation, electroporation, microinjection, liposome fusion, RBC ghost fusion, protoplast fusion, and the like.

For the transfection of a collection of cells, a library of vectors of interest, wherein each vector comprises only one copy of a nucleic acid molecule encoding one member of a recombinant polyclonal protein of interest, is used. This library of expression vectors of interest collectively encodes the recombinant polyclonal protein of interest.

The host cell line to be used for expression and production of a recombinant polyclonal protein of interest has one or more nucleic acid molecule(s) recognizable by a recombinase enzyme(s) (e.g. cells prepared beforehand having an FRT site at a pre-determined location in the genome as described in e.g. US 5,677,177).

The vector for site-specific integration is preferably integrated in a predefined genomic locus that mediates high-level expression, a so-called hot spot.

If expression levels need to be increased, gene amplification can be performed using selection for a DHFR gene or a glutamine synthetase (GS) gene. This requires the use of vectors comprising such a selection marker.

A universal promoter cassette for constitutive expression having two promoters placed in opposite transcriptional direction, such as a head-to-head construction surrounded by the variable heavy chain and the whole of the kappa light chain was constructed, allowing transfer of the whole construct into a vector for site specific integration said vector comprising a FRT site and a hygromycin resistance gene. It is contemplated that a promoter cassette for inducible expression can also be used. Furthermore, the promoters can be placed tail-to-tail which will result in transcription in opposite direction or tail-to-head for unidirectional transcription. CHO-Flp-In cells (Invitrogen) which stably express the lacZ-Zeocin fusion gene, were used for the experiment, rendering the cells resistant to the antibiotic Zeocin. The cells were maintained in a medium containing Zeocin. The cells were transfected with the vector for site-specific integration containing a GOI and a different selection marker (hygromycin) together with a plasmid expressing the Flp recombinase. An inducible promoter can also be used for control of the expression. After transfection, the cells were cultivated in the presence of hygromycin. Cells that were resistant to hygromycin were subsequently grown in different culture systems, such as conventional small culture flasks, Nunc multilayer cell factories, small high yield bioreactors (MiniPerm, INTEGRA-CELLine) and spinner flasks to hollow fiber-and bioreactors. The cells were tested for protein production using ELISA. Cell lines were selected for viability in suspension growth in serum free medium without selection pressure for extended periods. Stocks of cell lines were grown in the presence of hygromycin.

Evaluation of the preservation of polyclonality in the expression system

To be able to evaluate the stability and reproducibility of the expression system, vectors encoding six distinct Fab fragments with reactivity against chicken ovalbumin (OVA), bovine alkaline phosphatase (AP), human β_2-microglobulin (β_2m), human haptoglobin (HAP), human Factor VIII (FVIII) and hen egg white lysozyme (LYS) were prepared. The different Fab fragment encoding sequences have different nucleic acid molecules and therefore exhibit different RFLP patterns, whereby RFLP can be used for analyzing the genotype composition.

The antibody-coding sequences from each clone were introduced into CHO-Flp-In cells by transfection using an expression vector with a head-to-head promoter cassette. The CHO-Flp-In cells were either transfected with a mixture of expression vectors of interest encoding the six distinct antibodies resulting in a cell line expressing the six antibodies in known genotype composition or the cells were transfected individually with one of the expression vectors of interest followed by mixing of the transfected cells, generating a recombinant polyclonal antibody expressing cell line expressing the six antibodies in known composition. In this manner, it was possible to test whether the transfection of the mammalian cells occurs without generating a bias to one or several individual clones of the recombinant polyclonal antibody expressing cell line. Furthermore, it was possible to check for growth bias and bias caused by the purification of the polyclonal composition of antibodies.

Establishment of an anti-ovalbumin recombinant polyclonal antibody manufacturing cell line

Ovalbumin-binding phage clones were selected using phage display and ELISA to identify the relevant clones. Two setups were used for identifying antibodies from the ovalbumin-binding clones, i.e. ELISA plates coated with ovalbumin or a high density screening method (HDS), based on immobilization of ovalbumin on PVDF membranes. In this manner a panel of antibodies were obtained, of which some recognize ovalbumin immobilized on the ELISA plate and others recognize ovalbumin immobilized on the PVDF membrane.

The selected ovalbumin-binding phage clones may have their variable heavy and kappa chain DNA sequences linked to mammalian promoters and transferred into a vector of the pSymvc20 type for antibody expression generating a collection of clones of the pSymvc21 type. The CHO-Flp-In cells are either transfected with a mixture of the pSymvc21 clones or the cells are transfected individually with one pSymvc21 antibody expressing plasmid followed by mixing of the transfected cells expressing the other ovalbumin binding antibodies. The procedure of creating an anti-ovalbumin polyclonal antibody producing cell line can be monitored by DNA sequencing, TaqMan PCR and RFLP analysis of individual antibody expressing cells, as well as ELISA, 2-dimensional (2D) liquid chromatography (LC) and mass spectrometry (MS) of the produced antibody mixture.

Cultivation of cells and production of a recombinant polyclonal antibody

The cell line produced as described above is grown in suitable media under suitable conditions for expressing the antibody encoded by the nucleic acid molecules inserted into the genome of the cells. After a suitable time, the expressed recombinant polyclonal antibody is isolated from the cells or the supernatant. The recombinant antibody is purified and characterized according to procedures known by a person skilled in the art. Examples of purification and characterization procedures are listed below.

Purification of a recombinant polyclonal protein from culture supernatant

Affinity chromatography combined with a second purification step such as gel filtration has frequently been used for the purification of IgG (polyclonal as well as monoclonal) from different sources e.g. ascites fluid, cell culture supernatants and serum. Affinity purification, where the separation is based on a reversible interaction between the protein(s) and a specific ligand coupled to a chromatographic matrix, is an easy and rapid method, which offers high selectivity, usually high capacity and concentration into a smaller volume. Protein A and protein G, two bacterial cell surface proteins, have high affinity for the F_c region, and have, in an immobilized form, been used for many routine applications, including purification of polyclonal IgG and its subclasses from various species and absorption and purification of immune complexes.

Gel filtration, as a final purification step, can be used to remove contaminant molecules such as dimers and other aggregates, and transfer the sample into storage buffer. Depending on the source and expression conditions it may be necessary to include an additional purification step to achieve the required level of antibody purity.

In order to purify other classes of antibodies, alternative affinity chromatography media have to be used since proteins A and G do not bind IgA and IgM. An immunoaffinity purification

can be used (anti-IgA or anti-IgM monoclonal antibodies coupled to solid phase) or, alternatively, multistep purification strategies including ion-exchange and hydrophobic interaction can be employed.

Structural Characterization

Structural characterization of polyclonal proteins such as antibodies requires high resolution due to the complexity of the mixture (clonal diversity and glycosylation). Traditional approaches such as gel filtration, ion-exchange chromatography or electrophoresis may not have sufficient resolution to differentiate among the individual antibodies. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) has been used for profiling of complex protein mixtures followed by mass spectrometry (MS) or LC-MS (e.g. proteomics). 2D-PAGE, which combines separation on the basis of a protein's charge and mass, has proven useful for differentiating among polyclonal, oligoclonal and monoclonal immunoglobulin in serum samples. However, this method has some limitations. Chromatographic techniques, in particular capillary and LC coupled to electrospray ionization MS are increasingly being applied for the analysis of complex peptide mixtures. LC-MS has been used for the characterization of monoclonal antibodies and recently also for profiling of polyclonal antibody light chains. The analysis of very complex samples requires more resolving power of the chromatographic system, which can be obtained by separation in two dimensions (or more). Such an approach could be based on ion-exchange in the first dimension and reversed-phase chromatography (or hydrophobic interaction) in the second dimension optionally coupled to MS.

Functional Characterization

Immunoprecipitation is a highly specific technique for the analytical separation of target antigens from crude cell lysates. By combining immunoprecipitation with other techniques, such as SDS-PAGE followed by protein staining (Coomassie Blue, silver staining or biotin labeling) and/or immunoblotting, it is possible to detect and quantify antigens e.g., and thus

evaluate some of the functional properties of the antibodies. Although this method does not give an estimate of the number of antibody molecules nor their binding affinities, it provides a visualization of the target proteins and thus the specificity. This method can likewise be used to monitor potential differences of the antibodies toward antigens (the integrity of the clonal diversity) during the expression process.

Therapeutic compositions

In an embodiment of the invention, the pharmaceutical composition according to the invention is one intended for the treatment or prevention of a disease in a mammal such as a disease selected from cancer, infections, inflammatory diseases, allergy, asthma and other respiratory diseases, autoimmune diseases, cardiovascular diseases, diseases in the central nervous system, metabolic and endocrine diseases, transplantation rejections and undesired pregnancy. The mammal is preferably a human, a domestic animal or a pet.

For the treatment or prevention of infections, the pharmaceutical composition according to the invention comprises a recombinant polyclonal protein of interest capable of reacting with or binding to an infectious microorganism such as a microorganism selected from bacteria, mycobacteria, virus, mycoplasma, rickettsia, spirochetes, protozoa, fungi, helminthes and ectoparasites.

Recombinant human polyclonal antibodies may be administered within a pharmaceutically-acceptable diluent, carrier, or excipient, in unit dosage form. Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer the compounds to patients suffering from a disease, for example, caused by excessive cell proliferation. Administration may begin before the patient is symptomatic. Any appropriate route of administration may be employed, for example, administration may be parenteral, intravenous, intra-arterial, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, suppository, or oral administration. For example, therapeutic formulations may be in the form of, liquid solutions or suspensions; for oral administration, formulations may be in the form of

tablets or capsules chewing gum, pasta, compositions suitable for the application onto the skin may be in the form of creams, ointments, lotions, gels, pads or other, compositions suitable for application onto the vaginal or urogenital mucosa may be in the form of vagitories, gels or other and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

The pharmaceutical compositions of the present invention are prepared in a manner known *per se*, for example, by means of conventional dissolving, lyophilising, mixing, granulating or confectioning processes. The pharmaceutical compositions may be formulated according to conventional pharmaceutical practice (see for example, in Remington: The Science and Practice of Pharmacy (20th ed.), ed. A.R. Gennaro, 2000, Lippincott Williams & Wilkins, Philadelphia, PA and Encyclopedia of Pharmaceutical Technology, eds. J. Swarbrick and J. C. Boylan, 1988-1999, Marcel Dekker, New York, NY).

Solutions of the active ingredient, and also suspensions, and especially isotonic aqueous solutions or suspensions, are preferably used, it being possible, for example in the case of lyophilized compositions that comprise the active ingredient alone or together with a carrier, for example mannitol, for such solutions or suspensions to be produced prior to use. The pharmaceutical compositions may be sterilized and/or may comprise excipients, for example preservatives, stabilisers, wetting and/or emulsifying agents, solubilisers, salts for regulating the osmotic pressure and/or buffers, and are prepared in a manner known per se, for example by means of conventional dissolving or lyophilising processes. The said solutions or suspensions may comprise viscosity-increasing substances, such as sodium carboxymethylcellulose, carboxymethylcellulose, dextran, poly vinylpyrrolidone or gelatin. The injection compositions are prepared in customary manner under sterile conditions; the same applies also to introducing the compositions into ampoules or vials and sealing the containers.

Pharmaceutical compositions for oral administration can be obtained by combining the active ingredient with solid carriers, if desired granulating a resulting mixture, and processing the mixture, if desired or necessary, after the addition of appropriate excipients, into tablets, drage

cores or capsules. It is also possible for them to be incorporated into plastics carriers that allow the active ingredients to diffuse or be released in measured amounts.

The pharmaceutical compositions comprise from approximately 1% to approximately 95%, preferably from approximately 20% to approximately 90%, active ingredient. Pharmaceutical compositions according to the invention may be, for example, in unit dose form, such as in the form of ampoules, vials, suppositories, drages, tablets or capsules.
The formulations can be administered to human patients in therapeutically effective amounts (e.g., amounts which prevent, eliminate, or reduce a pathological condition) to provide therapy for a disease or condition. The preferred dosage of therapeutic agent to be administered is likely to depend on such variables as the type and extent of the disorder, the overall health status of the particular patient, the formulation of the compound excipients, and its route of administration.

If desired, treatment with recombinant human polyclonal antibodies may be combined with more traditional therapies. For example in the treatment of cancer such combinatorial therapies could take the form of surgery or administration of chemotherapeutics or other anti-cancer agents.

In another embodiment of the invention, the pharmaceutical composition according to the invention comprises a recombinant polyclonal protein of interest capable of reacting with or binding to an infectious microorganism such as a microorganism selected from bacteria, mycobacteria, virus, mycoplasma, rickettsia, spirochetes, protozoa, fungi, helminthes and ectoparasites.

Therapeutic uses of the compositions according to the invention

The pharmaceutical compositions according to the present invention may be used for the treatment or prevention of a disease in a mammal. Diseases that can be treated with the present pharmaceutical compositions include cancer, infectious diseases, inflammatory diseases, allergy, asthma and other respiratory diseases, autoimmune diseases, cardiovascular

diseases, diseases in the central nervous system, metabolic and endocrine diseases, transplantation rejections and undesired pregnancy.

Diagnostic use and environmental detection use

Another embodiment of the invention is directed to diagnostic kits and kits for environmental detection use as well as methods for using these kits. Kits according to the present invention comprise a recombinant polyclonal protein prepared according to the invention which protein may be labeled with a detectable label or non-labeled for non-label detection. If labeled, the present recombinant polyclonal protein may be added to a sample suspected of containing the target molecule and the presence or absence of the label indicate the presence or absence of the target molecule. The sample to be tested may be a sample of bodily fluid such as blood, serum, plasma, spinal fluid, lymph or urine or a non-mammalian sample such as a sample from an environmental source suspected of harboring a contaminent. Non-mammalian samples may be water, air or contaminated earth. Non-label detection encompasses the measurement of refractive change in BIAcore upon binding, wherein the recombinant polyclonal protein is used to capture the target molecule.

Examples

The following examples describe how recombinant polyclonal antibodies are expressed and produced in a high producer cell line, where gene(s)/vector(s) of interest have been inserted by site-specific integration into a pre-characterized chromosomal "hot spot" site.

In the examples, CHO cells were utilized as host cell. The advantages thereof include the availability of suitable growth medium, their ability to grow efficiently to a high density in culture, plus their ability to express mammalian proteins such as immunoglobulin in a biologically active form.

In general, transformation of *E. coli* and transfection of mammalian cells according to the subject invention will be performed according to conventional methods. To improve the

understanding of the invention, construction of exemplary vectors and their usage in producing a manufacturing cell line for recombinant polyclonal protein expression are described in the examples below.

The following examples illustrate the invention, but should not be viewed as limiting the scope of the invention.

Example 1: Site-specific integration versus random integration

For the following transfection experiment, the CHO Flp-In cells (Invitrogen) were used. The efficiency of the system was tested using human secreted alkaline phosphatase (SEAP) as a reporter gene. Two plasmid constructs were prepared:

1. SEAP inserted into pcDNA3.1hygro+ (Invitrogen) (for random integration)
2. SEAP inserted into pcDNA5/FRT (Invitrogen) (for site-specific integration)

The two plasmid constructs were very similar with respect to regulatory elements, i.e. promoter, polyadenylation etc. which made it possible to use the plasmids for comparing random integration with site-specific integration.

CHO Flp-In cells were transfected with plasmid construct 1 alone or plasmid construct 2 together with the recombinase-encoding plasmid pOG44 according to the procedure described by Invitrogen. Transfectants were selected using hygromycin and the production of SEAP from pools of transfectants was measured.

Cells transfected by site-specific integration produced approximately 6 times more SEAP than cells transfected by random integration proving the efficiency of the system and the cell line.

Example 2: Design and preparation of an expression vector for site-specific integration in a host cell

An expression vector suitable for site-specific integration into a hot spot chromosomal region of a host cell may be assembled comprising the following DNA elements:

a) A FRT recombination site linked to the hygromycin resistance gene
b) A pUC origin of replication
c) An ampicillin resistance gene (bla)
d) A bla-promoter allowing expression of the ampicillin (bla) resistance gene
e) Gene(s), encoding a protein of interest (GOI('s))
f) Promoter(s) allowing expression of the GOI('s)
g) Optionally additional transcriptional or translational regulatory elements, such as enhancers or UCOE's, for increased expression at the site of integration or an IRES

To provide a better understanding of the construction of the expression vector, each of the elements are described in more details:

a) An FRT recombination site linked to the hygromycin resistance gene for Flp recombinase-mediated integration and selection of a cell line with a majority of single integrants was used. The hygromycin gene was neither preceded by a promoter nor equipped with a transcription initiating codon, but a polyadenylation signal was added 3′ of the gene. The FRT site used was (5′-gaagttcctattccgaagttcctattctctagaaagtataggaacttc-3′).

b) A pUC origin of replication was included to permit high copy number replication in an *E. coli* host cell.

c) An Ampicillin (bla) resistance gene (β-lactamase) allowing selection of *E. coli* transformants was included.

d) A bla-promoter allowed expression of the ampicillin (bla) resistance gene in *E. coli*.

e) GOI encoding a protein of interest, e.g. a recombinant polyclonal protein, immunoglobulin, the heavy and light chains of an antibody, as well as nucleotide sequences that encode all or a

portion of either the constant region or variable region of an antibody molecule, and optionally all or a portion of a regulatory nucleotide sequence that controls expression of an antibody molecule were included.

Immunoglobulin loci for heavy chains may include but is not limited to all or a portion of the V, D, J and switch region (including intervening sequences, also known as introns) and flanking sequences associated with or adjacent to the particular heavy chain constant region gene and it may include regions located within or downstream of the constant region (including introns).

Immunoglobulin loci for the light chains may include but are not limited to the V and J regions, their upstream flanking sequences, and intervening sequences (introns) associated with or adjacent to the light chain constant region gene, and it may include regions located within or downstream of the constant region (including introns).

For the modification of all or a portion of a constant region of an antibody, modifying sequences of the invention may include, but are not limited to an immunoglobulin constant region having a particular effector function, class and/or origin (e.g. IgG, IgA, IgM, IgD, or IgE constant regions of a human immunoglobulins or any other species) or a portion of a constant region which modifies the activity or properties of the constant region of the immunoglobulin; as well as genes which encode other molecules that confer some new function to a modified antibody molecule, e.g. an enzyme, toxin and the like.

The gene(s) encoding a protein of interest may be operatively linked to nucleotide sequences encoding functional leader sequences directing the gene product to the secretory pathway. Further, 3' of the GOI encoding the protein of interest, e.g. such as a polyclonal antibody comprising heavy and light chains, there may be strong polyadenylation signals. The use of the mouse isotype IgG1 in the following examples is for illustrative purposes and is not intended to limit the scope of the invention.

f) Promoters allowing expression of the GOI are provided. Therefore, a cassette comprising promoter and enhancer elements for expression is described. In the expression vector, each of the antibody genes may be preceded by their own mammalian promoter elements directing high level expression of each of the two chains, whether uni-directionally, bi-directionally or a tail-to-tail orientation of transcription cassettes is used.

In a bi-directional orientation of expression, a head-to-head promoter configuration can be used (construction of such a system is described in details in US 5.789.208, which is incorporated by reference in its entirety). In a uni-directional expression system, two promoters or one promoter combined with e.g. an IRES sequence can also be used for expression.

For construction of head-to-head promoters, a Pfu PCR amplification of the promoters is performed individually. The 5'-primer will initiate on the 5' most base of the promoter, the 3'-end primer will include a unique restriction site such as, for example, a *Xba*I site. Following PCR amplification, the fragments may be separated on an agarose gel, and isolated from the gel using QiaQuick columns (Qiagen). This is followed by an *Xba*I restriction digestion, heat inactivation at 65°C for 20 minutes, and column purification of the fragments using QiaQuick. The fragments are then mixed and ligated together using *E. coli* ligase (New England Biolabs (NEB)), an enzyme that preferentially ligates sticky ends. The ligation mix is PCR amplified with the 5'-primers of each promoter to yield the complete head-to-head promoter (promoter A / promoter B) fragment. This fragment is kinased with T4 polynucleotide kinase (PNK) (NEB), the enzyme is heat inactivated at 65°C for 20 minutes, and the fragment is ligated (blunt end) into the vector of interest (PCR amplified pSymvc10 (see Figure 3) fragment, where the primers used for amplification anneal on each side of the promoter region amplifying everything except the promoter) using T4 ligase (NEB). Figures 1 and 2 show expression vectors comprising promoters for bi-directional and uni-directional, respectively. These promoters intend to illustrate, but not limit, the promoter choice in the invention.

g) The expression vector can carry additional transcriptional and/or translational regulatory elements, such as enhancers and/or UCOE's, for increased expression at the site of integration and/or IRES.

Example 3: Evaluation of polyclonality preservation in the manufacturing system developed

In order to be able to evaluate the stability and reproducibility of the manufacturing system, a cell line expressing a polyclonal composition of distinct antibodies in known composition was prepared.

(a) Clone origin

The following sequences encoding Fab fragments (the genes of interest) with reactivity against antigens 1-6 were used in this example:

1. Ovalbumin (OVA). The Fab encoding fragments were selected from a murine anti-OVA phage display library.

2. Alkaline phosphatase (AP). The Fab encoding fragments were selected from a murine anti-AP phage display library.

3. β_2-microglobulin (β_2m). The Fab encoding fragments were cloned from the hybridoma BBM.1 (a gift from Dr. L. Ø. Pedersen, Denmark), which was generated against β_2m.

4. Human haptoglobin (HAP). The Fab encoding fragments were selected from a murine anti-human haptoglobin phage display library.

5. Factor VIII (FVIII). The parental monoclonal antibody of this Fab fragment was a FVIII F25 monoclonal antibody (gift from Novo Nordisk, Denmark). The DNA encoding the V_H and complete Kappa chains of this Fab fragment was sub-cloned into a phagemid, followed by insertion of the prokaryotic promoter cassette into the construct.

6. Hen egg lysozyme (LYS). This construct was generated from the D1.3 scFv clone (Boulot, G. et al., J. Mol. Biol., 213(4) (1990) 617-619), by PCR amplification of V_H and V_K fragments and cloning into a phagemid.

The phagemid clones exist either in transformed *Escherichia coli* strain TG1 glycerol stocks (kept at -80°C) or as phagemid DNA preparations.

(b) RFLP analysis and DNA sequencing

The nucleotide sequence encoding the heavy chains of the Fab fragments were analyzed by RFLP as follows: The band patterns obtained after digest of the PCR generated fragments with the *Nla*III and *Hinf* I enzyme were examined. The different Fab fragment encoding sequences exhibited very different and easily distinguishable patterns. The nucleotide sequences encoding the V_H and V_L fragments were sequenced and sequences corresponding to the RFLP pattern were found. Furthermore, the nucleotide sequences encoded open reading frames and translated into well-defined polypeptides.

(c) ELISA analysis

The Fab fragments expressed from the clones were analyzed in a criss-cross ELISA, in which all Fab fragments were analyzed for reactivity with all antigens. Fab expression was monitored using an anti-Kappa ELISA. All Fab fragments were tested in duplicate in ELISA. All clones expressed Fab fragments, and the Fab fragments reacted specifically with their relevant antigen. No background problems were found in the ELISA analyses.

The six phagemid clones exist in individually transformed *Escherichia coli* strain TG1 glycerol stocks, which were used in the model system for inoculation described below.

(d) Design of a polyclonal model system with six distinct antibodies in known composition

The six selected Fab-expressing clones (clones expressing Fab fragments of anti-OVA, anti-AP, anti-β_2m, anti-HAP, anti- FVIII, and anti-LYS) were characterized by testing the reactivity of the expressed Fab fragments against the relevant antigens. These clones formed part of a polyclonal model system for testing the expression and production of six distinct

antibodies in a known composition. All Fab fragments encoding nucleotide sequences were cloned into a phagemid vector (illustrated by pSymvc10, Figure 3 A).

(d.1) Individual transfer of the GOI's from the phagemid vector into a vector for mammalian expression

The transfer of the genes of interest from a phagemid vector to a vector for mammalian expression was, in this example, performed in a two-step procedure. The first step was to replace the prokaryotic promoters with a mammalian promoter cassette in a head-to-head orientation. This step was followed by transferring the variable region of the GOI's, the promoter cassette and the constant kappa to the expression vector as described in detail below, and illustrated in Figure 3.

The head-to-head promoter cassette (promoter A / promoter B) was inserted into the phagemid vector for each clone by using a *SacI*/*XhoI* digestion followed by a ligation resulting in change of promoters from bacterial to mammalian. An *Eco*RI and *NotI* digest was then used to move the variable heavy chain, the head-to-head promoter cassette (promoter A / promoter B) and the complete kappa chain (*Eco*RI /Not I fragment) from the phagemid vector into the expression vector.

An example on the individual transfer of each clone is given with the flow chart in Figure 3. This Figure shows plasmid pSymvc10 where the heavy and kappa coding sequences of interest (e.g. gc032 OVA) are present in the phagemid vector into which the head-to-head mammalian promoter cassette construct was ligated to replace the bacterial promoters using a *SacI*/*XhoI* fragment transfer generating pSymvc12.

From this construct, the variable heavy chain-coding sequence including the promoter cassette and the whole of the kappa chain coding sequence was transferred into the mammalian isotype-encoding vector (pSymvc20) by a *NotI*/*Eco*RI transfer. The resulting vector (pSymvc21) expressed the mouse antibody of interest (e.g. anti-OVA IgG1 antibody).

The variable heavy chain coding sequence, the mammalian promoter cassette and the whole of the kappa chain coding sequence from each of the six clones were transferred individually by a *NotI*/*Eco*RI transfer resulting in the mammalian expression vector pSymvc21, which expresses each of the GOI encoded antibody sequences as mouse IgG1 antibodies.

The six individual pSymvc21 clones containing the six genes of interest were kept as TG1 glycerol stocks.

For transfection into CHO-Flp cells, the TG1 stocks were propagated individually, and after OD_{600} normalization for the number of *E coli* cells, the six cultures were mixed and used for plasmid preparation. This plasmid preparation comprising the six genes of interest was used to transfect mammalian cells for recombinant polyclonal protein expression, here represented as a polyclonal model system with six distinct antibodies in known composition.

(d.2) Mass transfer of the GOI's from phagemid vectors into vectors for mammalian expression

The GOI's (here the *Eco*RI/*Not*I fragments), which was located in phagemid vectors and coding for six distinct Fab fragments (anti-OVA), anti-AP, anti-β_2m, anti-HAP, anti-FVIII, and anti-LYS), were mass transferred as a mixture of the six vector constructs and into vectors for mammalian expression resulting in a mixture of six distinct expression vectors.

The experimental procedure concerning the mass transfer follows the procedure described in (d.1) with the exception, that it was performed in mass, i.e. all six GOI (encoding the variable heavy chains, including the head-to-head promoter cassette and complete kappa chains) were transferred simultaneously as a mixture from the phagemid vectors.

Plasmid preparations

Plasmid Preparation 1 refers to a plasmid preparation of a mix of the six phagemid vectors (with the antibody coding sequences contained in the vector pSymvc10).

Plasmid Preparation 2 refers to a plasmid preparation of six phagemid vectors with the coding sequences contained in the vector pSymvc12 after mass transfer step 1, which results in the exchange of the prokaryotic promoters with the mammalian promoter cassette constructs.

Plasmid Preparation 3 refers to a plasmid preparation after mass transfer step 2, which affords exchange of the variable heavy chain, the head-to-head promoter cassette, and the complete kappa chain from the pSymvc12 to the mammalian expression vector (pSymvc21), thus allowing expression of the six selected antibodies as mouse IgG1 antibodies.

Genotyping of TG1 cells transformed with plasmid preparations used in mass transfer

TG1 cells were transformed by electroporation and after an overnight incubation on 2x YT (Sigma Y 2627) plates, individual colonies were picked. In each experiment 180 colonies were picked and incubated in 96 well formats in 2xYT liquid medium for 4 hours. Aliquots of the cultures were diluted with water, denatured and used as template in PCR. In all experiments, the variable heavy chain was amplified. Primer sequences for the phagemid vectors (pSymvc10-type) were:

5'-GCATTGACAGGAGGTTGAGGC-3' and

5'-GCTGCCGACCGCTGCTGCTGGTC-3'

Primers for vectors with mammalian promoter cassette were (pSymvc12-type):

5'-GCATTGACAGGAGGTTGAGGC-3' and

5'-GTGTCCACTCTGAGGTTCAG-3'

Primers for pSymvc21 constructs were:

5'-CAAATAGCCCTTGACCAGGC-3' and

5'-GTGTCCACTCTGAGGTTCAG-3'

All PCR products were digested with both *Nla*III and *Hin*fI to ensure unambiguous genotyping. The digestion fragments were analyzed by agarose gel electrophoresis and bands were visualized by EtBr staining. The number of individual genotypes resembled by the fragment pattern determined by RFLP corresponds to the number of individual colonies among the total number of picked colonies.

(d.2a) Mass transfer from the phagemid vector to a mammalian vector after DNA amplification in *E. coli* cells

The Plasmid Preparation 1 was prepared from each of the six *E. coli* TG1 glycerol stocks, containing one of the six phagemid vectors. The stocks were propagated individually, and after OD_{600} normalization for the number of *E. coli*, the six cultures were mixed in equal amounts and used for plasmid preparation resulting in the preparation Plasmid Preparation 1. The genotype distribution of the six phagemid vectors in Plasmid Preparation 1 was tested by transformation into electrocompetent TG1 cells and subsequent RFLP analysis. The distribution of the different genotypes in TG1 cells is shown in Figure 4.

The Plasmid Preparation 1 comprising the polyclonal phagemid vector expressing an equal mixture of the six selected Fab fragment genotypes was digested with *SacI/Xho*I. Then the head-to-head promoter cassette (CMV promoter/MPSV promoter) was inserted by ligation. The genotype distribution of the vectors after the promoter exchange in the vector was tested in TG1 cells after transformation with DNA from the ligation step (Figure 5).

The cells were plated and grown on large (245mm x 245mm) 2x YT agar plates and the Plasmid Preparation 2 was prepared to generate the phagemid vector now containing the head-to-head promoter cassette (pSymvc12).

From the Plasmid Preparation 2, the variable heavy chain coding sequence, including the promoter cassette and the whole of the kappa chain sequence was cut out from the phagemid vector by a *NotI/Eco*RI digest and transferred into a vector (pSymvc20) already containing the

constant region domains of mouse IgG1. This resulted in a collection of pSymvc21 vectors, which expresses the variable region of the six selected antibody clones as full-length mouse IgG1 antibodies.

The promoter transfer can alternatively be performed in the mammalian vector encoding an isotype, reversing the order of restriction digest starting with *NotI/EcoRI* for transfer of the DNA of interest to the mammalian vector and then *SacI/XhoI* restriction digest fragment for insertion of the promoter region.

The distribution of genotypes after transferring the variable heavy chain coding sequence, the promoter cassette and the whole kappa chain encoding sequence into the expression vector was tested by transforming TG1 cells with DNA from the second ligation step (Figure 6). Cells were plated on large (245 mm x 245 mm) 2x YT agar plates and Plasmid Preparation 3 was prepared (pSymvc21), in which the variable region of the six clones are expressed as mouse IgG1 antibodies).

The Plasmid Preparation 3 can be used to transfect mammalian cells for recombinant polyclonal antibody expression.

The results of the mass transfer from the phagemid vector to an isotype-encoding mammalian vector after DNA amplification in *E. coli* cells showed that it was possible to obtain a balanced distribution of the six vector constructs after propagated individually and mixed (Plasmid Preparation 1, Figure 4). The six constructs, after exchange of promoter cassette (Plasmid Preparation 2, Figure 5) as well as after insertion into a mouse IgG1 isotype-encoding vector (Plasmid Preparation 3, Figure 6), were all detectable at comparable levels.

(d.2b) Mass transfer from phagemid vector to a vector for mammalian expression without DNA amplification in *E. coli* after the Plasmid Preparation 1 step

DNA from the Plasmid Preparation 1 (here was used 25 μg) comprising the polyclonal phagemid vector (pSymvc10) expressing an equal mixture of the six selected Fab fragment

genotypes was digested with *SacI/XhoI* for exchange of promoters. The *SacI/XhoI* vector fragment was purified and ligated with the head-to-head promoter cassette (CMV promoter/MPSV promoter). After the exchange of promoters without performing any amplification, the vector with CMV/MPSV promoter cassette was digested with *NotI/Eco*RI for cutting out the whole region with the variable heavy chain encoding sequence, including the promoter cassette and the whole of the kappa chain encoding sequence from the phagemid vector for mass transfer into a vector for mammalian expression. After ligating the *NotI/Eco*RI fragment encoding the variable heavy chain, the promoter cassette and the kappa chain into a mouse IgG1-encoding vector (pSymvc20), an expression vector, which expresses the variable region of the six selected clones in the context of mouse IgG1 full length antibodies was obtained. The composition of this expression vector is illustrated in Figure 1.

After the mass transfer resulting in the promoter exchange in the vector and transfer of the nucleotide sequences encoding the variable heavy chain, the promoter cassette and the whole of the kappa chain into a vector for mammalian expression, the distribution of genotypes was tested by transformation of TG1 cells with plasmid from the second ligation step. Cells were plated on large (245 mm x 245 mm) 2xYT agar plates and a plasmid preparation of this double digestion/ligation Plasmid Preparation was prepared (vector pSymvc21, in which the variable region of the six clones are expressed as mouse IgG1 antibodies, corresponding to Plasmid Preparation 3 from d.2a). The genotype distribution in TG1 cells after transformation with the double digestion/ligation plasmid preparation is shown in Figure 7. This genotype distribution shows a minor random skewing of the frequency of the individual antibodies, as compared with results presented in Figure 6.

The product from the double digestion/ligation plasmid preparation (the purified mammalian expression vector pSymvc21, in which the coding sequences of the variable region from the six selected phagemid clones are expressed as mouse IgG1 antibodies) can be used directly to transfect mammalian cells for recombinant polyclonal antibody expression.

The known antibody composition of this polyclonal model system can be used to test and ensure that the mass transfer and transfection into mammalian cells occurs without bias in the

composition of the antibody variable sequence genotypes during transfection and subsequent culture. The methods by which the genotypic composition will be monitored throughout the process of mass transfer and mammalian expression, can comprise the following:

- DNA sequencing of isolated clones
- RFLP analysis of individual clones
- ELISA of the produced antibody mixture
- Mass spectrometry of the produced antibody mixture
- Taqman PCR of the relative composition of the genomic sequences and mRNA expressing the different heavy and light chains

These methods will enable the analysis of the genotype composition of the six distinct antibodies in known composition throughout the whole process.

(e) Expression of the six distinct antibodies in known composition in mammalian cells

After transferring the six selected GOI from the phagemid vector to the mammalian expression vectors, either individually as described in (d.1) or by mass transfer as described in (d.2), the mammalian expression vectors were used for transfection into a hot spot in a CHO-Flp-In cell line by using site-specific integration for expressing the six distinct antibodies as described below.

For the individually transferred GOI (d.1), plasmid DNA's were propagated individually and used for individual transfection into CHO-Flp cells or the TG1 stocks were propagated individually, and after OD_{600} normalization for numbers of *E. coli* cells, the six cultures were mixed and used for plasmid preparation. This plasmid preparation containing the six genes of interest was used to transfect mammalian cells for recombinant polyclonal antibody expression.

For the mass transferred GOI (d.2a or d.2b) plasmid DNA's are either transfected into CHO-Flp-In cells or amplified and purified (Plasmid Preparation 3) according to the procedure described in (d.2a or d2.b) and then used for transfection.

(e.1) Cell culture

The CHO Flp-In host cell line (Invitrogen) was maintained in Ham's F-12 medium, with the addition of glutamine (2 mM) and FCS (10%) and 100 µg/ml Zeocin (Invitrogen). For sub-culturing, the cells were detached by trypsin and split according to manufacturer's instructions. Cells were grown at 5% CO_2, 37°C. Medium and medium additives were from Gibco.

This cell line stably expresses the lacZ-Zeocin fusion gene, rendering the cells resistant to the antibiotic Zeocin, a resistance that upon site-specific integration of a foreign gene will be lost. The cells contain a single copy of the Flp Recombination Target (FRT) site, and are thus ready to be used as host cell line for site-directed integration by use of the Flp-In system (Invitrogen).

(e.2) Transfection of CHO cells

Tissue culture plates with 6 wells were inoculated with 4.0×10^5 CHO-Flp-In cells/ well, and incubated O/N at 37°C / 5 % CO_2. Transfection of these cells was performed testing different transfection methods using FuGENE™6 (Roche), Lipofectine™, LipofectAmine™, or LipofectAMINE 2000™ (Gibco) according to the manufacturer's instructions. In this example, LipofecAMINE 2000™ was used as transfection reagent. Briefly, on the day before the transfection, exponentially growing CHO-Flp-In cells were seeded as described above and incubated O/N at 37°C / 5 % CO_2. Wells with an 85-95 % cell confluence were used for the co-transfection.

Two tubes with the following contents were prepared:

Tube 1) 0.5 µg of an individual expression vector with GOI described in (d.1) (e.g. pSymvc21 with OVA) + 4.5 µg mp040 (a maxi preparation of pOG44) (a plasmid expressing recombinase Flp) were added to 250 µl Optimem (1.5 ml Eppendorf tubes).

Tube 2) 7.5 µl LipofectAMINE 2000™ was added to 250 µl Optimem (1.5 ml Eppendorf tubes) and incubated at room temperature (RT) for 5 min.

The content of tube 2) was transferred to tube 1) followed by incubation at RT for 20 min.

The DNA-Lipofectamine complexes were transferred to the wells with cells according to the manufacturer's instructions.

After 24 h, the cells were detached by trypsin, split (1:3) and distributed to a T-25 flask and to 100 mm petri dishes and cultivated in fresh Nutrient Mixture F-12 Ham + 10% FCS + 2 mM L-Glutamine medium with 900 µg Hygromycin B/ml as selection pressure.

(e.3) Selection of site-specific integrants and sub-cultivation of transfected cells

Cells were cultivated under Hygromycin selection pressure for two to three weeks, in this period cells were refreshed every 2 to 4 days with new medium containing the same concentration of selecting agent. The surviving pool of cells in the T-25 flask and in Petri dishes were detached by trypsin, split (1:6) and distributed to T-flasks for further propagation under the above mentioned selection pressure. Some single clones were picked (using so-called cloning cylinders) from the Petri dishes containing the transfectants generated according to the method described in (d.1), and transferred to new wells for propagation and use in expression level studies.

Each pool of cells or single clones, that is resistant to the threshold Hygromycin B concentration, was subsequently grown to confluence in 6-well plates, reseeded to Petri dishes, T-25, T-80 and T-175 flasks in their respective medium plus Hygromycin B. When

exponentially growing cells reach 80% confluence in T80 tissue culture flasks, vials of each cell line was frozen and stored in liquid nitrogen N_2(L)- freezer.

For transfection with a mixture of plasmids containing the six genes of interest, the six individual cultures were normalized at OD_{600} for numbers of *E. coli*, mixed and used for a polyclonal plasmid preparation containing the six genes of interest. A transfection procedure using 7.5 times as much of the reagents and cells described above was carried out, producing a recombinant polyclonal cell line expressing a mixture of the six distinct antibodies.

The six cell lines expressing each of the selected antibodies and the cell line expressing the mixture of the six distinct antibodies were during cultivation and propagation tested for antibody production by antigen-specific ELISA.

(f) Cell line expressing six distinct antibodies in known composition

By generating a mixture of the six selected genes of interest situated in expression vectors followed by transfection and site-specific integration into CHO-Flp-In cells, a cell line expressing six distinct antibodies was generated.

The cell line was followed for 34 days in which genotype distribution, antibody expression and proliferation rates were followed.

(f.1) Genotype distribution of the six selected genes of interest in CHO-Flp-In cells transfected with the plasmid preparation from the OD_{600} normalized mixture of cells.

CHO-Flp-In cells were trypsinized and the cell suspension diluted to 10 cells/ml. Hereafter, 200 µl were transferred to wells in plates with 96-wells in a total of 10 plates. Approximately 10 days later, wells with single colonies were identified by microscopy. Wells with single colonies were washed 1x in PBS and 50 µl water was added. Plates were incubated at 80 °C

for 10 min and lysates were transferred to another 96-well plate. Ten µl of the lysates were used in 25 µl OneStep RT-PCR (Qiagen) with the following primers:

5'-CAAATAGCCCTTGACCAGGC-3' and
5'GTGTCCACTCTGAGGTTCAG-3'

RFLP was performed using *Hin*fI and *Nla*III on 10 µl of RT-PCR mixtures in 15 µl reactions that were incubated at 37 °C for 2 hours. The digestion fragments were visualized using agarose gel electrophoresis followed by EtBr staining of the gel. The genotype distribution of cells producing anti- OVA, anti-AP, anti-β_2m, anti-HAP, anti-FVIII, and anti-LYS was followed over time (days 16 and 34 after transfection), see Figure 8.

(f.2) ELISA of samples derived from CHO-Flp-In cells transfected with the plasmid preparation from the OD_{600} normalized mixture of *E. coli* (d.1)

CHO-Flp-In cells were trypsinized and 3 x 10^6 cells were plated in T-75 flasks in F-12 HAM + 10% FCS + 2 mM L-glutamine and 900 µg hygromycin. Medium was changed every day and at day 3 supernatants were selected for ELISA. Antigens, (β2-microglobulin (a gift from University of Copenhagen), alkaline phosphatase (Sigma), ovalbumin (Sigma), factor VIII (a gift from Novo Nordisk, Denmark), hen egg white lysozyme (Sigma), and haptoglobin (Sigma)) were diluted in 50 mM carbonate buffer to 10 µg/ml. ELISA plates were coated with antigen (50 µl to each well) and incubated O/N at 4 °C. Wells were washed 4 times with washing buffer (1x PBS/0.05% Tween 20) and blocked for 1 hour with 2% skim milk powder in washing buffer (100 µl to each well). 50 µl samples were added to the wells and plates incubated for 1 hour at RT. Plates were washed 4x and secondary antibodies (Goat anti-mouse IgG/HRP conjugate (Sigma)) were added for 1 hour followed by 4x wash. The ELISA was developed with TMB substrate (50 µl in each well, DAKO S1600) for 5 min and reactions stopped by adding 50 µl 1 M H_2SO_4. Plates were read immediately at 450 nm. Data demonstrating expression of all six antibodies of interest in lysates derived on day 34 post-transfection with the mixture of expression vectors encoding the six genes of interest is shown

in Figure 9. It should be noted that since the data presented in Figure 9 is derived from six different antigen-specific ELISA assays, the OD450 readings are not directly comparable in terms of antibody quantity.

Antibody expression in pools of CHO-Flp-In cells transfected with individual GOI or the mixture of the six GOI is shown in Figures 9 and 10.

(g) Conclusions from the experiment

Firstly, these evaluations (tests) of preservation of the polyclonality in the manufacturing system showed that mass transfer of the six selected genes of interest (encoding anti-OVA, anti-AP, anti-β_2m, anti-HAP, anti-FVIII, and anti-LYS) from phagemid vectors into mammalian expression vectors was possible without introduction of selection or growth bias (Figures 7), thereby ending up with comparable frequency of the six selected genes of interest.

Secondly, transfection of CHO-Flp-in cells with a mixture of the constructs containing the six selected genes of interest also resulted in comparable distribution of the constructs in isolated mammalian cells. The genotype distributions of the six selected genes of interest over time (day 16 and 34 after transfection) were also similar (Figure 8), indicating that the expression system up to day 34 maintained the original, equal distribution of the six genotypes, without introducing growth bias.

Thirdly, the cells transfected with the mixture of the six genes of interest showed expression of all six antibodies, as examined by antigen-specific ELISA on supernatants from cells 34 days after transfection (Figure 9). The ELISA results for the different antigens are not directly comparable in terms of antibody amounts, due to different binding affinities. However, a capture ELISA based on coating with goat anti-mouse kappa chain antibody performed on supernatants from a) the six transfected CHO-Flp-In cell lines generated using the vector preparation as described in (d.1) and b) on supernatants of the cell line expressing a mixture

of the six selected genes of interest showed comparable antibody expression levels from the six genotypes.

In summary, as has previously been demonstrated (Sharon et. al) it is feasible to transfer a polyclonal GOI in mass from a phagemid vector to a mammalian expression vector. Furthermore, a mixture of mammalian expression constructs could be transfected into mammalian cells and maintained at a comparable frequency at least up to day 34 post-transfection.

Example 4: Establishment of an anti-ovalbumin recombinant polyclonal antibody manufacturing cell line

(a) Expression of an anti-ovalbumin polyclonal antibody composition

A collection of fully characterized ovalbumin-binding phage clones has been identified as follows. Four eight-week old female BALB/c mice were immunized i.p. and s.c. with 50 µg OVA in complete Freunds Adjuvant and boosted with OVA in incomplete Freunds adjuvant at days 21 and 42 after immunization day 0, and it was confirmed that all animals had sero converted against OVA, as measured by an antigen-specific ELISA. Spleens were harvested from the best responding mice at days 31 and 52. Fab-displaying phagemid libraries were generated from splenic RNA, using the phagemid vector (Symvc10) as previously described. The resulting libraries contained approximately 10^6 independent clones. Selection of these libraries was performed by reacting 5×10^{11} Fab-displaying phagemids with OVA coated on NUNC immunotubes, followed by washing and acid elution of binding phages. As eluates from the first round of panning contained a significant proportion of OVA binders, eluates from first and second rounds of panning were screened for OVA-binding phage clones.

Initially, OVA-reactive phage clones were identified by ELISA. In brief, ELISA plates were coated with OVA and reacted with the phage-displayed Fabs, followed by an HRP-conjugated secondary antibody. For negative controls, irrelevant antigens (BSA) or irrelevant phage-displayed Fabs (anti-AP) were used.

In addition, a HDS method, based on OVA immobilization on PVDF membranes, was established. These two methods resulted in the identification of separate subsets of clones, i.e. some clones that recognized OVA in one set-up and not in the other and vice versa. For Fab-displaying phage clones that reacted with OVA by either ELISA or HDS, the nucleotide sequence encoding the variable domain of the V_H was determined by DNA sequencing, and the genetic diversity was estimated by phylogenetic analysis, using the Vector NTI software package. The resulting panel includes 127 OVA-binding clones, for all of which the nucleotide sequences of the variable part of the V_H have been established.

Fab fragments expressed by the 127 OVA-binding clones all have the ability to bind to ovalbumin either in native or denatured form. From this set we have identified approximately 30 different clones contained in a phagemid vector, e.g. pSymvc10, to be used for mass transfer and mammalian expression. These antibodies are expressed either in the form of mouse IgA, IgG2A or IgG2B antibodies. Because we have fully characterized the DNA sequences of these antibody producing clones, we are able to monitor the distribution of the genotypes throughout the mass transfer and mammalian expression procedure using the same methods as used for the model system with the six distinct antibodies described in example 3.

(b) Mass transfer of the OVA-specific antibody sequences to a vector for mammalian expression

The transfer of genes of interest from a phagemid vector to an expression vector is a two step procedure (illustrated in Figure 3), where the first step, is change of promoters with the promoter cassette with head-to-head orientation of the selected promoters, this is followed by transferring the variable region of the genes of interest and the promoter cassette to an expression vector. The head-to-head promoter cassette (promoter A/ promoter B) can be inserted into the phagemid vector of each clone by using a *SacI/XhoI* digest followed by a ligation resulting in change of promoters from bacterial to mammalian promoter (pSymvc12).

An *Eco*RI and *Not*I digest will then move the sequences encoding the variable heavy chain, the head-to-head promoter cassette (promoter A/ promoter B) and the complete kappa chain from the phagemid vector (pSymvc12) into an isotype-encoding vector, pSymvc20. The pSymvc20 vector can accept any *NotI/Eco*RI fragment from phagemid vector. This fragment would transfer the sequence encoding the variable heavy chain to connect with the constant heavy chain sequences in pSymvc20 as well as the entire sequence encoding the kappa chain to be connected with BGH PolyA sequence. This mass transfer will result in expression vectors as shown in Figure 1, which express the variable heavy regions and the entire kappa chains as mouse IgG2B antibodies after the mass transfer.

The vector, pSymvc20, can contain the mouse constant regions of the heavy chain of the IgA, IgG2A, IgG2B, IgE or IgG1 genes, and is thus capable of expressing any of the relevant mouse immunoglobulin isotypes of choise.

(c) Expression of an anti-ovalbumin recombinant polyclonal antibody

By mass transfer, the sequences encoding the variable region of the heavy chain, the promoters and the entire kappa chain are moved from a phage vector library to isotype-encoding vectors resulting in a polyclonal mammalian expression vector composition. This is followed by transfection and site-specific integration into a CHO-Flp-In cell line, generating a recombinant polyclonal antibody manufacturing cell line. This latter cell line is generated by targeting the gene of interest encoding each member of the recombinant polyclonal protein into the same specific location in the genome of each transfected cell, and at the same time integrating only one copy of the expression construct containing said nucleic acid molecule in each transfected cell.

The cell cultures and the transfection and selection procedure is the same as described in example 3 (e.1-e.3).

(d) monitoring composition stability

To ensure that the mass transfer and transfection into mammalian cells occurs without introduction of considerable bias in regard to cloning, expression and diversity among the individual clones the process of mass transfer and mammalian expression can be monitored with the following methods:

1) Analysis of generation time of the pools of cells from each transfected construct
2) Analysis of expression level of the pools of cells from each transfected construct
3) Analysis by RFLP on single cells
4) ELISA of the produced antibody mixture
5) Mass spectrometry of the produced antibody mixture
6) Analysis by Taqman PCR on a defined batch size using V region-specific primers to identify ratio's of each different clone.
7) Analysis of the batch over time cultivated with and without selection pressure (hygromycin) was performed for the following parameters
   a) clonal distribution
   b) protein expression levels (quantity and distribution
   c) genomic stability
   d) effects of adaptation to serum-free media.

(e) Production of an anti-ovalbumin recombinant polyclonal antibody composition

The recombinant polyclonal antibody producing CHO-Flp-In cell line is grown in different culture systems, including conventional small culture flasks, Nunc multilayer cell factories, and small high yield bioreactors (MiniPerm, INTEGRA-CELLine). Further, the cell lines are adapted to serum free suspension for subsequent cultivation in spinner flasks, hollow fibers, and bioreactors.

The media used to grow the selected cell lines is serum free, protein free or chemically defined media as recommended by the manufacturer (Invitrogen, B&D, Hyclone).

Supernatants from attached or suspension cells that are cultured without selection (hygromycin) are collected. The collected supernatants are analyzed and characterized as described (3f). Production yields, functionality, and quality of the produced antibodies are checked during and after growth of the cells under fed batch or perfusion conditions. Cells in suspension are used for inoculation of larger spinner flasks or bioreactors.

The polyclonal antibody from the collected supernatants is purified for later use in animal studies.

Other embodiments and uses of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered exemplary only, with the true scope and spirit of the invention being indicated by the following claims.

Claims

1. A method for the manufacture of a recombinant polyclonal protein of interest, said method comprising:

a) providing a collection of cells, wherein a majority of individual cells comprises one nucleic acid molecule encoding a distinct variant member of said polyclonal protein, and said nucleic acid molecule being integrated in the same site of the genome of said individual cells;

b) culturing said collection of cells under conditions suitable for expression of said polyclonal protein; and

c) obtaining said expressed recombinant polyclonal protein from said collection of cells or cell culture media, preferably from the culture media.

2. A method according to claim 1, wherein said collection of cells, which collectively expresses the recombinant polyclonal protein of interest, is obtainable by

a) transfecting cells with a library of vectors of interest for site specific integration, where each member of said library of vectors comprises one nucleic acid molecule encoding one member of a polyclonal protein of interest, and

b) optionally selecting for cells comprising an integrated copy from said library of vectors of interest.

3. The method of claim 1, wherein said collection of cells is obtainable by:

a) transfecting separate aliquots of cells with each distinct member of a library of vectors of interest, wherein each member of said library comprises one nucleic acid molecule encoding one member of a polyclonal protein;

b) optionally selecting for cells comprising an integrated copy from said library of vectors of interest; and

c) pooling said transfected cells to form said collection of cells, which collectively expresses said recombinant polyclonal protein of interest.

4. The method of claim 2 or 3 wherein said library of vectors of interest comprises nucleic acid molecules encoding multiple members of a polyclonal protein.

5. The method according to any of the claims 1 to 4, wherein said nucleic acid molecule is operably linked to a heterologous regulatory sequence capable of expressing said nucleic acid molecule in a constitutive or inducible manner.

6. The method according to any of the claims 1 to 5, wherein said nucleic acid molecule is integrated in a locus that mediates high-level expression.

7. The method of claim 6, wherein said high-level expression is mediated from a heterologous regulatory sequence.

8. The method according to any of the claims 1 to 7, wherein each member of said polyclonal protein is comprised of two or more polypeptide chains.

9. The method according to any of the claims 1 to 8, wherein said polyclonal protein comprises antibodies or fragments thereof.

10. The method of claim 9, wherein said polyclonal antibody or fragment thereof comprises sequence differences between the individual antibody molecules in the variable region (V region) or in the constant region (C region) or both.

11. The method according to any of the claims 1 to 10, wherein said collection of cells is derived from a mammalian cell line or cell type,

12. The method of claim 11, wherein said mammalian cell line or cell type is selected from a group consisting of Chinese hamster ovary (CHO) cells, COS cells, BHK cells, myeloma cells, fibroblasts, HeLa, HEK 293, or PER.C6, or derivative cell lines thereof.

13. The method of claim 11, wherein said mammalian cell line is CHO cells.

14. A pharmaceutical composition comprising as an active ingredient, a recombinant polyclonal protein obtainable by a method according to any of the prciding claims and a pharmaceutically acceptable excipient.

15. The pharmaceutical composition of claim 14, wherein said recombinant polyclonal protein of interest is specific for or reactive against a predetermined disease target, preferably in a human, a domestic animal, or a pet.

16. The pharmaceutical composition of claim 15, wherein said disease target is selected from a group of molecules active in cancer, infection, inflammatory diseases, allergy, asthma and other respiratory diseases, autoimmune diseases, cardiovascular diseases, diseases in the central nervous system, metabolic and endocrine diseases, transplant rejection, and undesired pregnancy.

17. The pharmaceutical composition according to any of the claims 14 to 16, wherein said recombinant polyclonal protein of interest is capable of reacting with, or binding to an infectious microorganism.

18. The use of a recombinant polyclonal protein for the preparation of a composition for the treatment of diseases selected from a group consisting of cancer, infection, inflammatory diseases, allergy, asthma and other respiratory diseases, autoimmune diseases, cardiovascular diseases, diseases in the central nervous system, metabolic and endocrine diseases, transplant rejection, and undesired pregnancy.

19. The use of a recombinant polyclonal protein for the preparation of a composition for the treatment or prevention of an allergy.

20. A method for generating a recombinant manufacturing cell line, said method comprising:

a) providing a library of vectors of interest, each comprising one copy of a nucleic acid molecule encoding one member of a polyclonal protein of interest and one or more recombinase recognition sequences, said recombinase recognition sequence facilitating site specific integration into the genome of a manufacturing cell line;

b) introducing said library of vectors for site specific integration into a collection of cells, wherein each cell comprises one or more recombinase recognition sequences at specific sites in its genome; and

c) ensuring the presence in said cells of a suitable recombinase which is either (i) expressed by said cells into which said nucleic acid molecule is introduced, or (ii) operatively encoded by said vector of interest, or (iii) provided through expression from a second vector, or (iv) provided as a protein.

21. The method of claim 20, wherein a majority of the individual cells of said cell line are expressing a distinct variant member of a recombinant polyclonal protein of interest from said nucleic acid molecule, which is integrated into the same site of the genome of the individual cells of said manufacturing cell line.

22. The method according to one of the claims 20 or 21, wherein said library of vectors of interest for site-specific integration are introduced into a collection of cells by

a) transfecting said collection of cells with said library of vectors of interest; and

b) optionally selecting for cells comprising an integrated copy from said library of vectors of interest.

23. The method according to one of the claims 20 or 21, wherein said library of vectors of interest for site-specific integration are introduced into a collection of cells by:

a) transfecting separate aliquots of said cells with each member of said library of vectors of interest;

b) optionally selecting for cells comprising an integrated copy from said library of vectors of interest; and

c) pooling said cells, to form a collection of cells, wherein a majority of individual cells express a distinct variant member of a recombinant polyclonal protein of interest.

24. The method according to any of the claims 20 to 23, wherein said nucleic acid molecule is integrated into a locus that mediates high-level expression.

25. The method according to any of the claims 20 to 23, wherein each member of said polyclonal protein comprises two or more polypeptide chains.

26. The method according to any of the claims 20 to 25, wherein said polyclonal protein comprises antibodies, or fragments thereof.

27. The method according to any of the claims 20 to 26, wherein said collection of cells is derived from a mammalian cell line or cell type.

28. The method according to claim 27, wherein said mammalian cell line or cell type is selected from a group consisting of Chinese hamster ovary (CHO) cells, COS cells, BHK cells, myeloma cells, fibroblasts, HeLa, HEK 293, or PER.C6, or derivative cell lines thereof.

29. The method of claim 28, wherein said mammalian cell line is a CHO cell.

30. A recombinant manufacturing cell line comprising a collection of cells, wherein a majority of the individual cells are capable of expressing a distinct variant member of a recombinant polyclonal protein of interest from one copy of a nucleic acid molecule integrated into the same site of the genome of each cell in said collection of cells.

31. The recombinant cell line of claim 30, wherein said cell line is derived from a mammalian cell line or cell type.

32. The recombinant cell line according to claim 31, wherein said mammalian cell line or cell type is selected from a group consisting of Chinese hamster ovary (CHO) cells, COS cells, BHK cells, myeloma cells, fibroblasts, and immortalized human cells, preferred immortalized human cells are HeLa, HEK 293, or PER.C6, or derivative cell lines thereof.

33. The recombinant cell line of claim 32, wherein said mammalian cell line is a CHO cell.

34. A recombinant manufacturing cell line obtainable by a method according to any of the claims 20 to 29.

35. A library of vectors, wherein each vector comprises one or more recombinase recognition sequences and one copy of a nucleic acid molecule of interest encoding a distinct variant member of a recombinant polyclonal protein of interest.

36. The library of claim 35, wherein each member of said library of vectors further comprises a recombinase encoding nucleic acid molecule.

37. A recombinant polyclonal protein obtainable from a recombinant manufacturing cell line according to any of the claims 30 to 35.

38. The recombinant polyclonal protein of claim 37, wherein said protein comprises antibodies or fragments thereof.

Abstract

The invention relates to a method for manufacturing a recombinant polyclonal protein composition, in particular a recombinant polyclonal antibody composition. The method comprises obtaining a collection of cells, whereof a majority of the cells expresses a distinct variant member of the recombinant polyclonal protein composition from one copy of a nucleic acid molecule encoding one member of the final recombinant polyclonal protein composition. It is an important feature that the nucleic acid molecule be integrated into the same site of the genome of each cell. The cells are cultured under suitable conditions for expression of the polyclonal protein, which is obtained from the cells or culture supernatant. The nucleic acid molecule is introduced into the cells by transfection with a library of vectors for site-specific integration. The present method is suitable for manufacturing recombinant polyclonal antibodies, thereby making available a superior replacement of plasma-derived therapeutic immunoglobulin products.

Figure 1

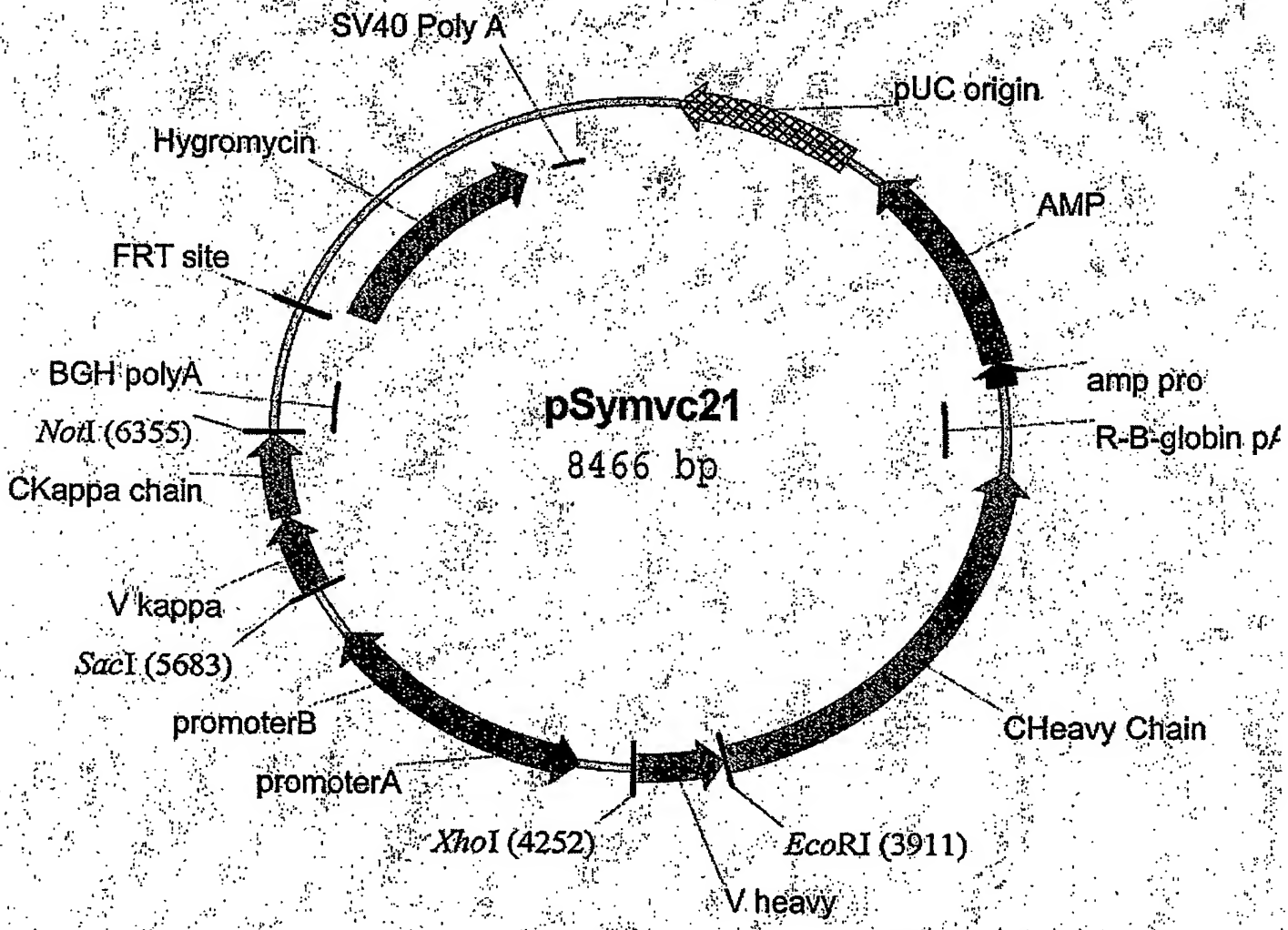

SV40 Poly A
pUC origin
Hygromycin
AMP
FRT site
amp pro
BGH polyA
R-B-globin pA
pSymvc21
8466 bp
NotI (6355)
CKappa chain
V kappa
SacI (5683)
promoterB
CHeavy Chain
promoterA
XhoI (4252)
EcoRI (3911)
V heavy

Figure 2

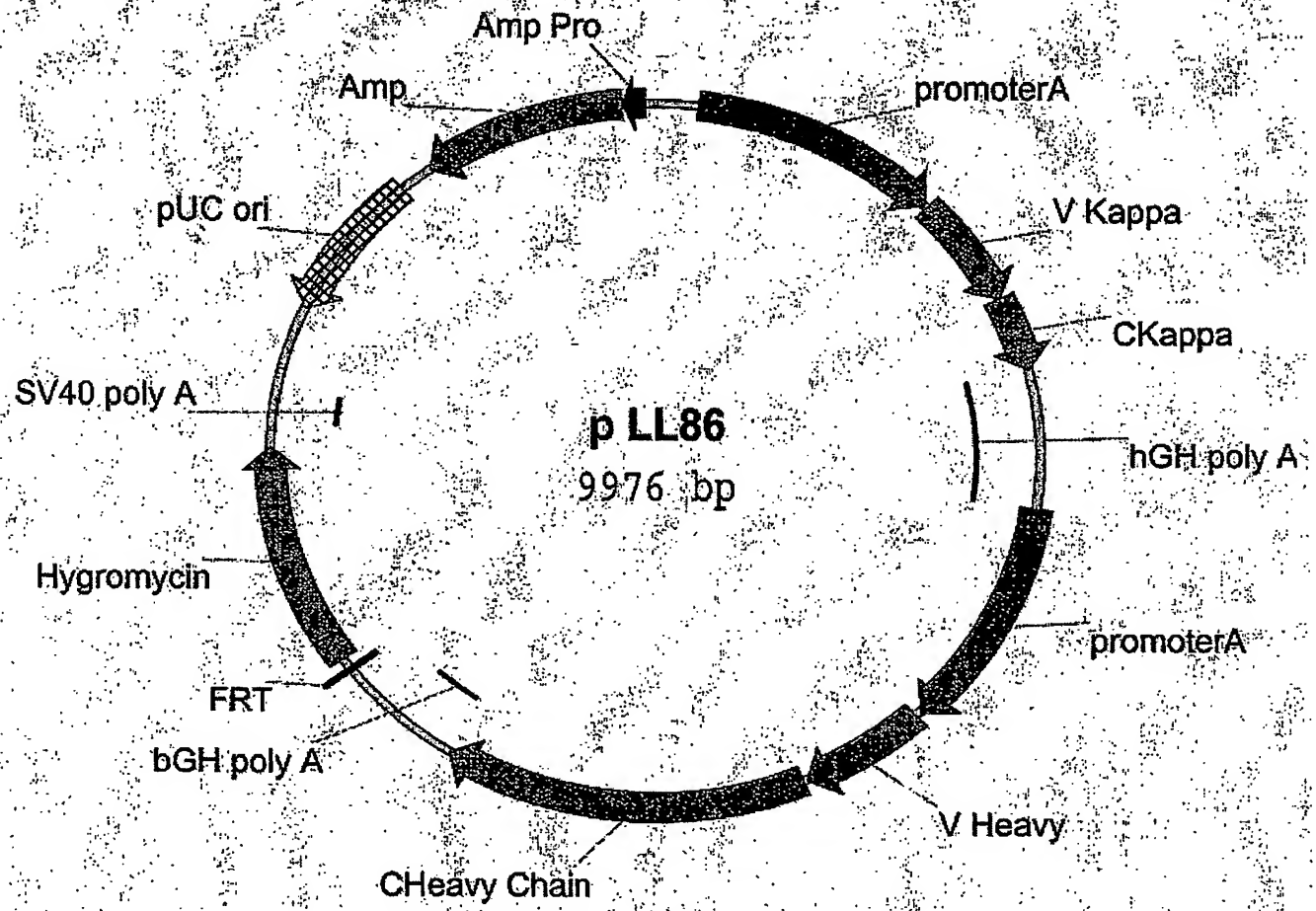
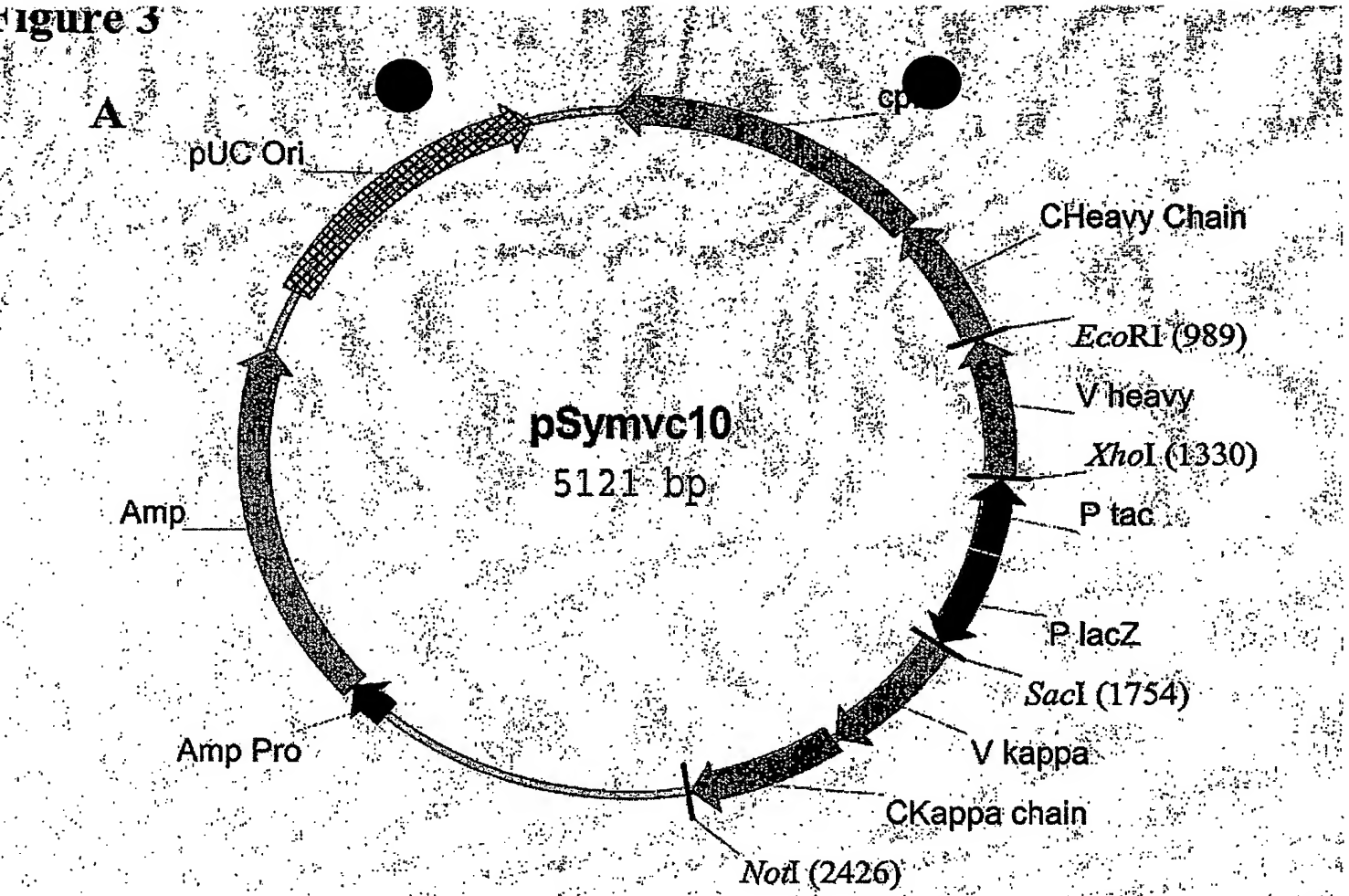


Figure 3



Fragment of promoter cassette (mammalian)
1433 bp (molecule 6135 bp)

Step 1 ↓ digest with SacI + XhoI,
ligate fragments from A and B, to generate
symvc12

Continued

Figure 3 continued

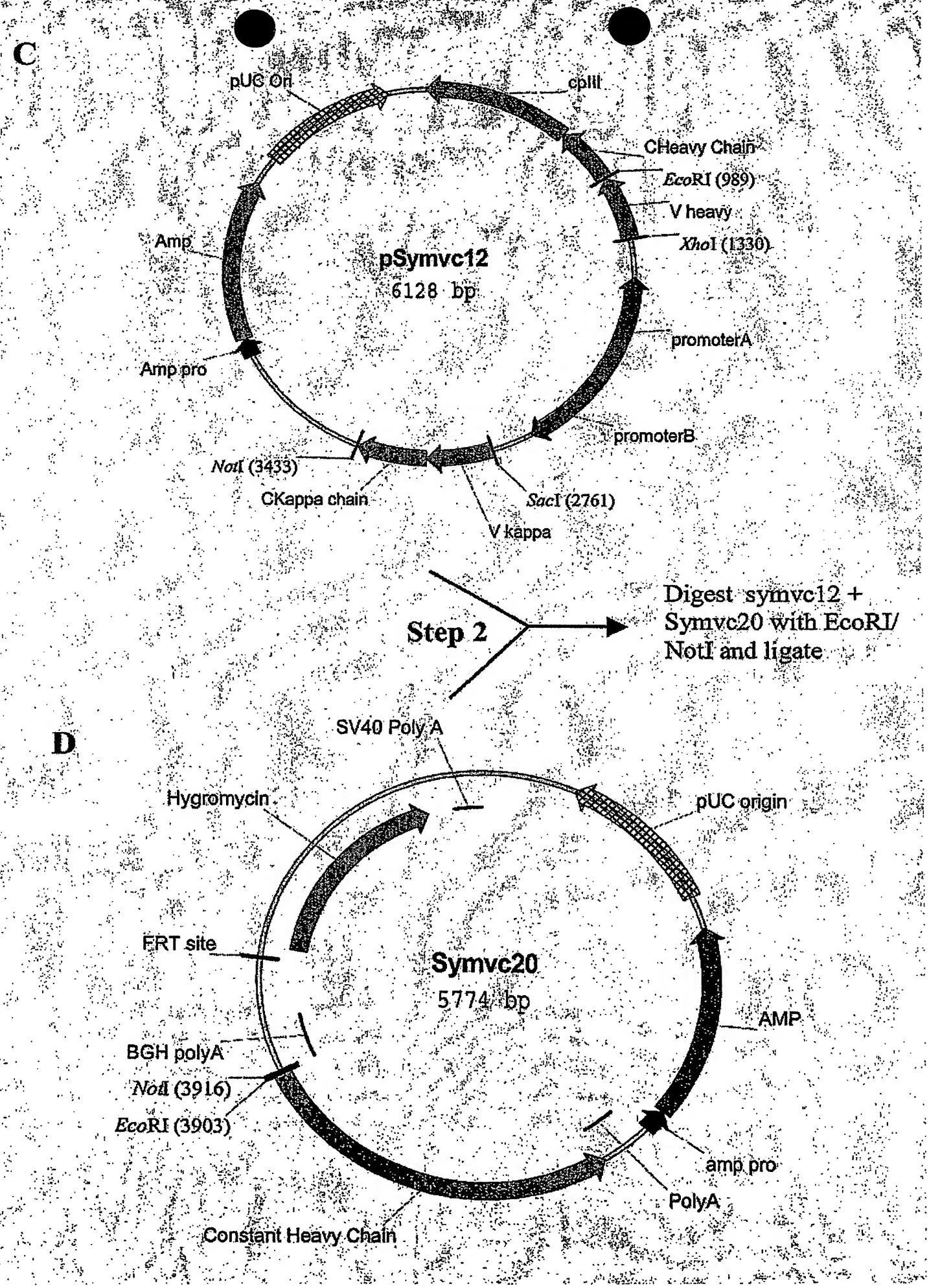


Figure 3 continued

E

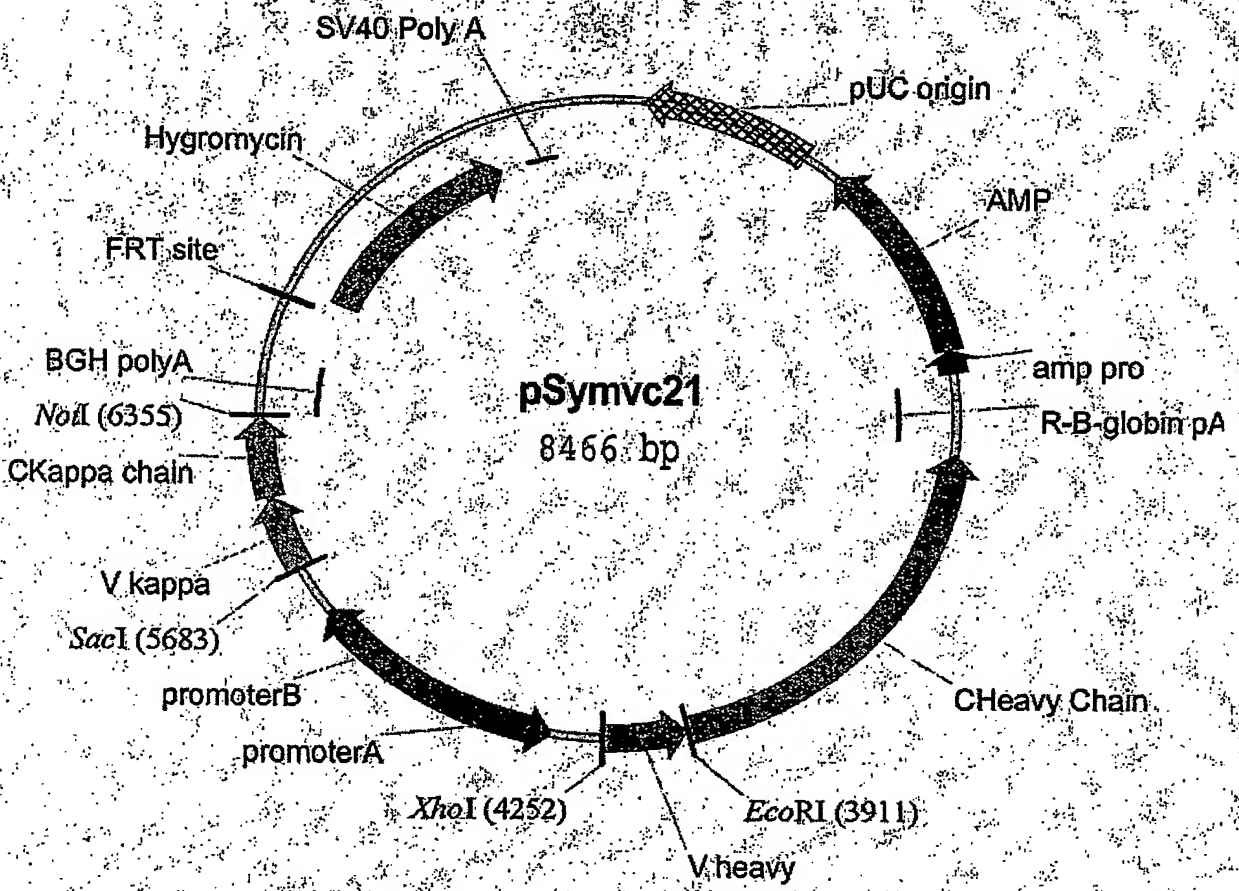


Figure 4

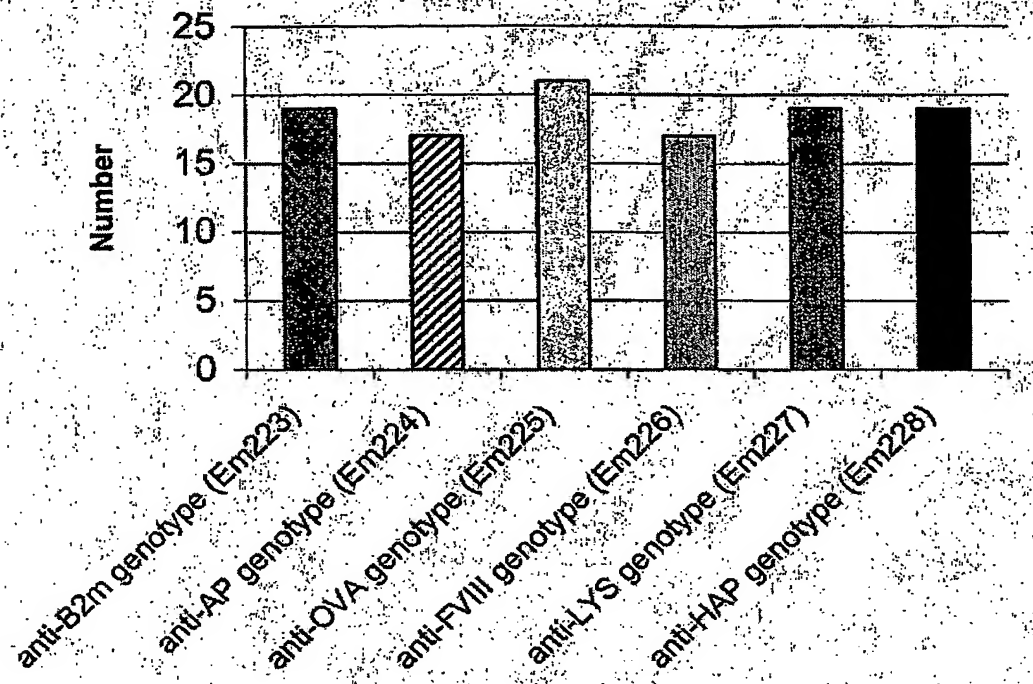


Figure 5

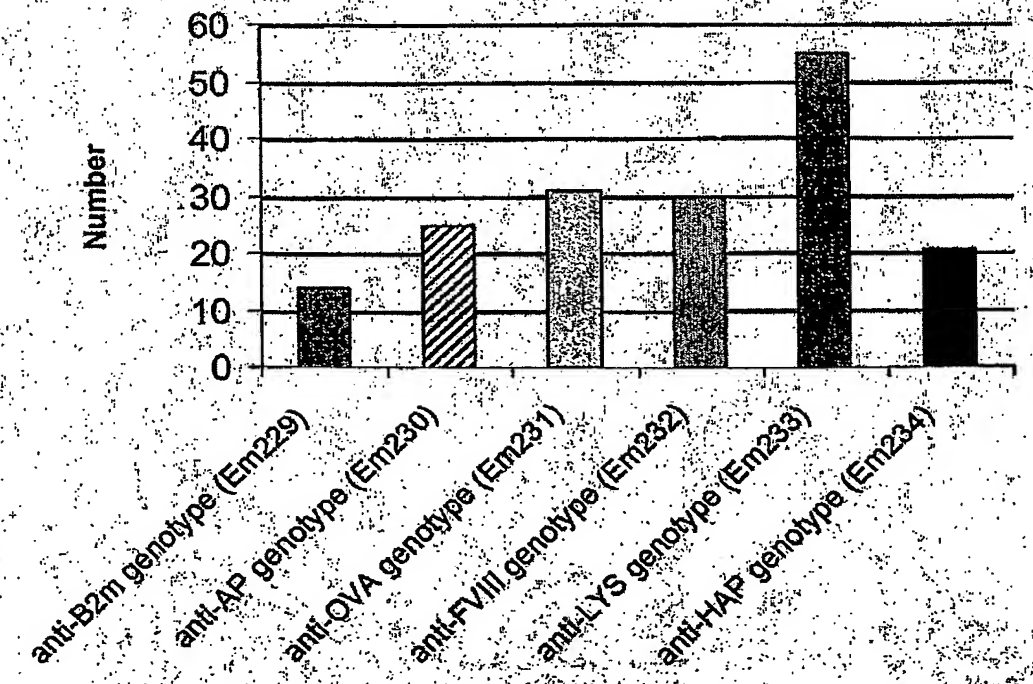


Figure 6

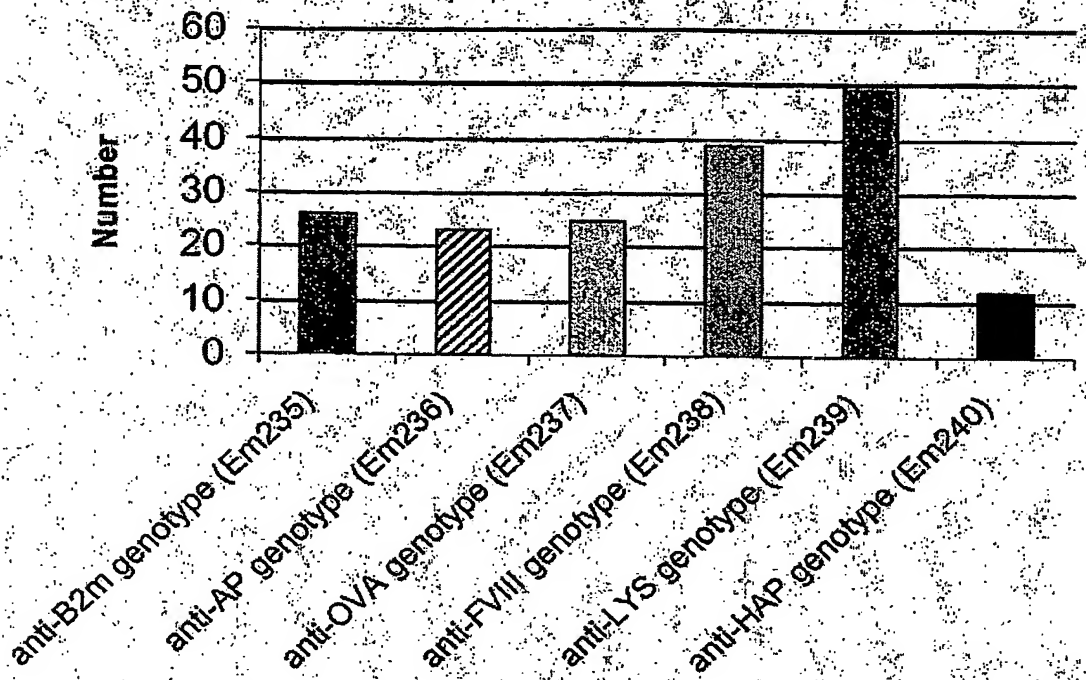


Figure 7

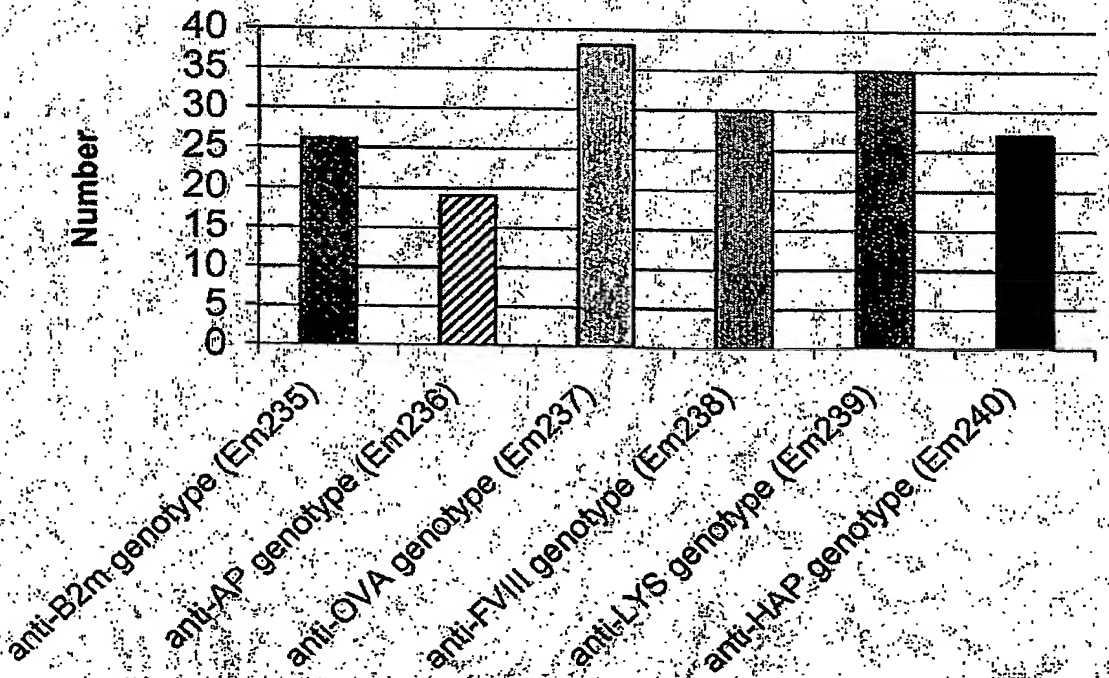
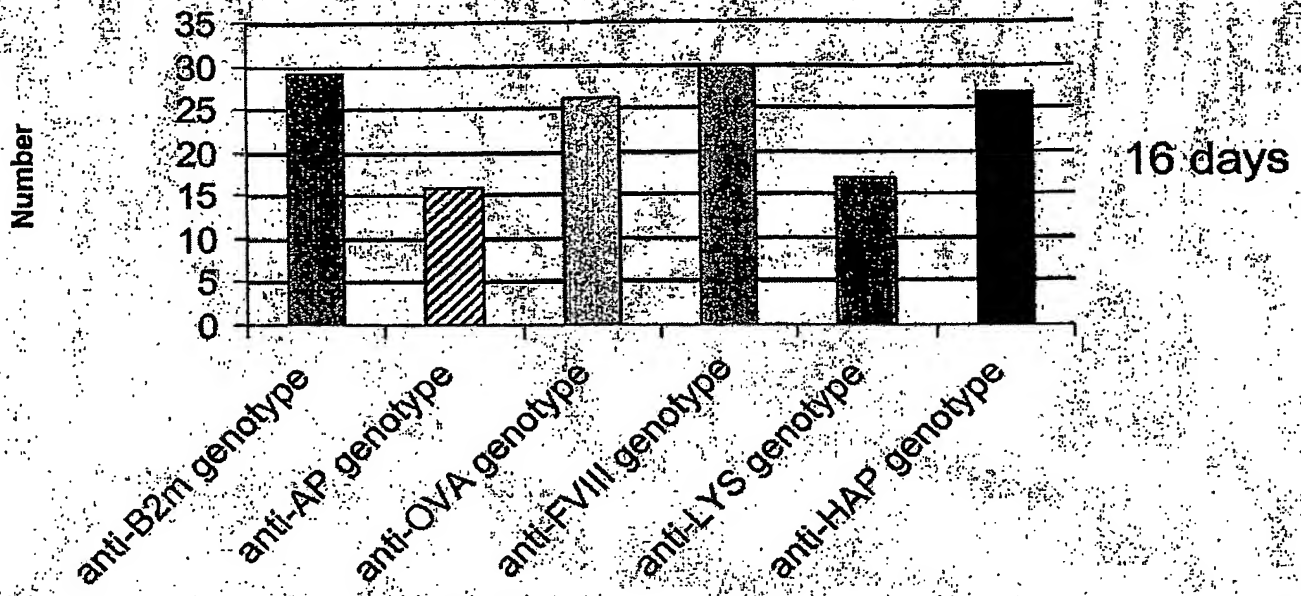


Figure 8

A


Number
35
30
25
20
15
10
5
0
16 days
anti-B2m genotype
anti-AP genotype
anti-OVA genotype
anti-FVIII genotype
anti-LYS genotype
anti-HAP genotype

B

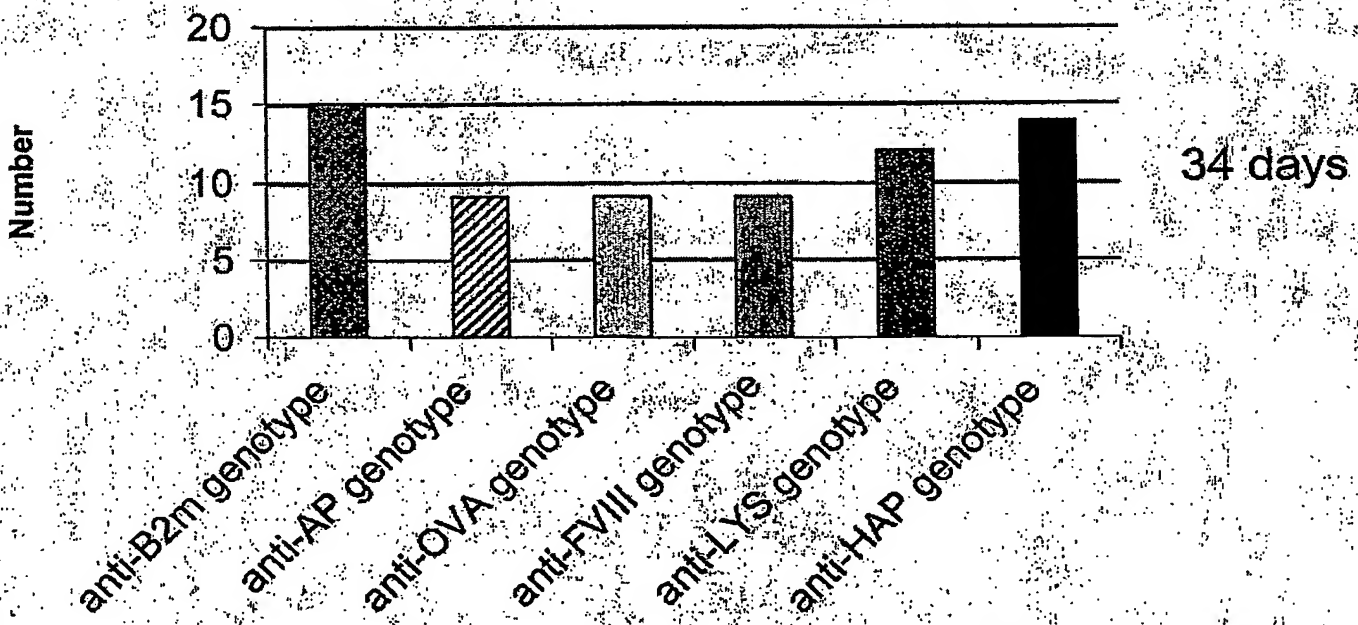

Number
20
15
10
5
0
34 days
anti-B2m genotype
anti-AP genotype
anti-OVA genotype
anti-FVIII genotype
anti-LYS genotype
anti-HAP genotype

Figure 9

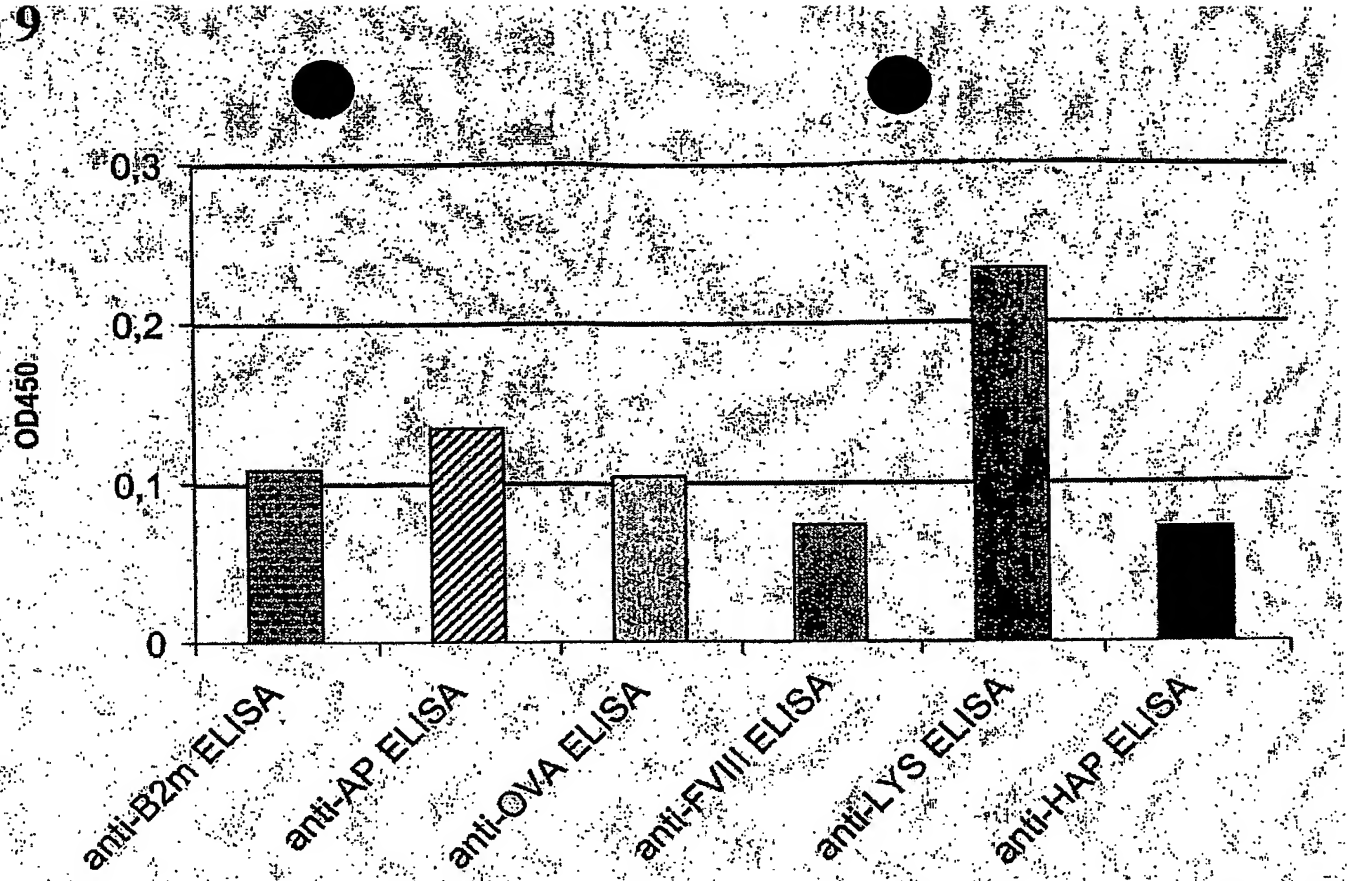


Figure 10